IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent application of: ROBERTSON, et al

No.: 09/380,327 Examiner: Huynh, Phuong N.

September 3, 1999 Art Unit: 1644

For: TREATMENT AND DIAGNOSIS OF INFERTILITY USING TGFBETA OR ACTIVIN Docket #: P07407US00/BAS

LETTER TO THE EXAMINER

Honorable Commissioner for Patents
Washington, D.C.

SIR:

In furtherance of the Amendment filed along with the CPA on December 23, 2002 in the above-identified case, Applicants now submit herewith the executed declaration of Dr. David Alexander Clark, M.D., Ph.D. along with the original exhibits.

In light of the submission of the executed document, in combination with the amendments and arguments as submitted on December 23, 2002, Applicants submit that the present case is in condition for immediate allowance, and such action is earnestly solicited.

Respectfully submitted,
LARSON & TAYLOR, PLC

January 10, 2003

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Declaration under U.S.C.§ Rule 132

IN THE MATTER OF
US Patent Application No.
09/380,327 by
ROBERTSON et al.

STATUTORY DECLARATION

I, David Alexander Clark of 444 Smith Ave., Burlington in the Province of Ontario, Canada, do solemnly and sincerely declare as follows:

1. I hold the degrees of Doctor of Medicine and Doctor of Philosophy, and am a Diplomate of the American Board of Internal Medicine, a Fellow of the Royal College of Physicians of Canada and a Fellow of the Royal College of Physicians of Edinburgh (UK). I am a Professor (now Emeritus) in the Department of Medicine, with cross appointment in the Departments of Obstetrics and Gynaecology, and Molecular Medicine and Pathology, in the Faculty of Health Sciences, McMaster University, Hamilton, Ontario, Canada, and a Professor in the Institute of Medical Sciences in the School of Graduate Studies of the University of Toronto. A copy of my *curriculum vitae* is now shown to me and annexed hereto as Exhibit DAC-1.

2. Since 1977, one of my major research interests has been the immunology of pregnancy, in particular the mechanisms underlying immunological rejection of the foeto-placental unit by the maternal immune system. I have published over 100 scientific papers in this field.

3. I am the same David A. Clark who is the first author of the paper entitled "$CD56^+$ lymphoid cells in human first trimester pregnancy decidua as a source of novel transforming growth factor-β_2" (Human Reproduction 1994 9 2270-2277). I am informed that this paper was cited by the Examiner in the Office Action dated 27 August 2002 issued in respect of this application. Other publications from my laboratory are referred to in the publication by Chaouat et al (J. Immunology 1985 134 1594-8), which was also cited in this Office Action.

4. I have read and understood the specification of the present application, US Application No. 09/380327, and the Office Action dated 27 August 2002 issued in respect of this application. I understand that this Office Action has been made final.

5. My understanding of the state of the field in relation to possible immunological causes of infertility and the role of TGFβ, as it stood at the priority date, 6 March 1997 is as follows:

Infertility in humans may occur due to
(i) failure of fertilization,
(ii) failure of the fertilized ovum to develop into a blastocyst,
(iii) failure of the blastocyst to implant,
(iv) failure during the period prior to implantation until formation of a vascularized placenta (i.e. occult losses, chemical pregnancies), or
(v) failure in the form of spontaneous abortion of a clinically evident pregnancy (i.e. missed menses + positive pregnancy test).

Immunological causes of (i) in 1997 included anti-sperm antibodies. and pro-inflammatory cytokines such as tumor necrosis factor α, IL-1, and interferon γ. Proinflammatory cytokines were found in the peritoneal fluid of women suffering from early endometriosis. This condition proceeds to fibrosis of the fallopian tubes and their ostia; TGFβ could contribute to such scarring. Concerning (ii), proinflammatory cytokines, particularly interferon γ, were proposed to arrest embryonic development. Problem (iii) could be produced by similar proinflammatory cytokines.

This is opposite to the teaching from the data of Robertson and Tremellen, in the present specification, which indicates that inflammation is important for implantation. However, proinflammatory cytokines were also viewed as deleterious to trophoblast outgrowth during nidation, and hence could contribute to (iv). TGFβs inhibited trophoblast outgrowth, and were viewed as potentially inhibitory to development. Indeed, Graham and Lala (J. Cell. Physiol. 1991 148 228-234) specifically showed that activation of TGFβ1 by trophoblast cells inhibited their invasive and proliferative properties. Autoimmunity in the form of autoantibodies, particularly antiphospholipid antibodies was thought important. Rote et al (Biol. Reprod. 1995 53 905-910 and 1997 56 50-58) suggested that certain antiphospholipid antibodies could bind to trophoblast cells, and block their ability to form a placenta. Gleicher et al (Am. J. Reprod. Immunol. 2002 48 252-254 and earlier in Am. J. Obstet Gynecol 1998 159 223-227) suggested that an polyclonal activation of autoantibody production occurred in those patients with autoimmunity and infertility, and is associated with activation of T cells producing proinflammatory Th1-type cytokines which are important in causing the infertility.

There was no information to suggest that any TGFβ isoform outside of the embryo *per se* was playing a positive role in fertility. The immunology of spontaneous abortion of an established pregnancy was thought to depend on inhibition of proinflammatory cytokine production by Th2/3 cytokines. The Th2 cytokine of particular importance was considered to be IL-10, although it is now known that IL-6 may also be important. A Th3 cytokine which was a TGFβ2-related molecule of novel structure and properties, which was disclosed in the paper by Clark et al cited by the Examiner, was viewed as particularly important. This cytokine was produced by cells bearing a γδ T cell receptor able to recognize antigens on trophoblast, unlike conventional T cells with αβ receptors, which are unable to trigger immune reactions to antigens on trophoblast. The Clark paper is discussed further below.

6. There was no information to support the idea that administering any conventional isoform of TGFβ could or would have any beneficial effect on any of the steps required for reproductive success.

7. I am the author of a review article, published in 1998, Clark DA, Coker R, Molecules in focus: Transforming growth factor-beta (TGF-β), Int. J. Biochem. Cell Biol. 1998 30 293-298), a copy of which is annexed hereto as Exhibit DAC-2. This was submitted on 30 July 1996, ie. before the claimed priority date of this application, and accepted on 3 October 1997. This review shows that at that time the only speculated diagnostic or therapeutic role for TGFβ in fertility pertained to the novel TGFβ2-related molecules described in Clark et al in recurrent spontaneous miscarriages.

A great deal has happened since 1997, and the only data indicating that exogenous administration of a conventional isoform of TGFβ could improve fertility has come from the research of Dr. Sarah Robertson, one of the inventors of the present application.

8. One of the Examiner's substantive objections is that the specification does not constitute an enabling disclosure, and includes subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the specification was filed, had possession of the claimed invention. I further understand that this objection results from the Examiner's opinion that the scope of the claims goes beyond what is disclosed in the specification.

9. In particular, the Examiner appears to consider that the specification provides an enabling disclosure only in respect of TGF-β_1, sperm antigens and MHC Class I, but does not encompass any other type of antigen, or any modified TGFβ, or analogue or fragment of TGFβ.

The full-length sequences of the different isoforms of TGF-β are known, and the sequences of a variety of fragments and analogues of various TGF-β isoforms are also known. I consider that the information provided in the specification would enable a person of ordinary skill in the art to determine whether any given fragment or analogue is suitable for use in the invention. In particular, intermediates in the processing pathway of activation of latent TGF-β could be used by a person of such ordinary skill to determine if they have the same fertility-enhancing and tolerance promoting effects as those demonstrated by the inventors.

10. I consider that it is clear from the introductory part of the specification, particularly the passage at page 4 line 14 to page 5 line 11 and from the summary of the invention at page 5 lines 25 to 28, that in order for a given molecule of the TGFβ superfamily, and in particular a TGFβ molecule, to be useful for the purposes of the claimed invention it must have the ability to induce synthesis of granulocyte-macrophage colony stimulating factor (GM-CSF) in uterine epithelial cells. TGFβ is a member of a "superfamily" of molecules, which is well recognised in the art, and which includes all of the isoforms of TGFβ, and other molecules such as activins, bone morphogenetic proteins, glial cell line-derived neurotrophic factor, and macrophage inhibitory cytokine-1. The specification as lodged therefore clearly demonstrates that TGFβ1, TGFβ2 and activin are all able to trigger the Type II response cascade. The activation of this cascade is known in the art to lead to the stimulation of antigen-presenting dendritic and macrophage cells, which subsequently invade the stromal tissue and increase the intensity of antigen signalling to the lymph nodes, all of which may participate in alleviation of an infertility condition.

11. I have been asked by the applicant's Australian patent attorney, Dr Vivien Santer of Griffith Hack, to consider the following questions:

a) At the priority date, 6 March 1997, would a person familiar with TGFβ have considered that it was reasonably predictable that TGFβ1, TGFβ2 and TGFβ3 would have similar effects in most situations?

It was known in March 1997 that different isoforms of TGFβ had similar actions in most situations. Any differences related to differences in concentration required to show the effects. For example, for TGF-β1 and β2 were immunosuppressive at a similar concentration, but suppression of bone marrow (myeloid) cell growth required a several-fold increase in TGFβ2 concentration compared to TGFβ1 (Nature 1987 329 539-541). Shah et al (J. Cell Sci. 1995 108 983-1002) had suggested that TGF-β3 might counteract the anti-fibrosis effects of TGF-β1, but to the best of my knowledge, these data have never been confirmed. During embryogenesis, distinct patterns of expression of different

isoforms had been noted. The meaning of such differences was a matter of speculation, since data from relevant knock-out mice had not been published.

b) Would such a person's opinion have changed between the priority date and date of publication, 11 September 1998?

I do not consider that this opinion would have changed during this period.

c) In particular, would TGFβ1, TGFβ2 and TGFβ3 have been reasonably expected to have a similar effect on fertility?

Caniggia et al (Endocrinology 1997 138 4977-4988) have shown that TGFβ1 and β3 bound to endoglin on trophoblast, and then would inhibit proliferation by binding to Type I+II receptors; TGF-β2 bound directly to the latter to produce this inhibition. There was no basis for thinking that conventional isoforms of TGFβ should act differently on fertility.

I therefore consider that at the claimed priority date, 6 March 1997, it was accepted in the art that all three isoforms of TGFβ have similar biological activities, especially in relation to immunosuppression.

12. I note that the present specification at page 6 lines 11 to 23 states that the three isoforms of TGFβ have 70 to 80% sequence homology, and share many biological actions. The passage at page 9 lines 7 to 9 states that

"the specificity of TGFβ to be coadministered with the male antigens is at present not entirely clear, and because $TGF\beta_1$ is thought to be responsible whereas $TGF\beta_{2,3}$ are less important, it is more likely that $TGF\beta_1$ is to be used."

However, the following passage, from page 9 lines 9 to 17, goes on to make it clear that the invention contemplates that TGFβ2, TGFβ3, activin, or other members of the TGFβ superfamily may also be used, and that the invention encompasses modifications of these molecules.

13. The experiments reported in the specification at pages 16 to 29 mainly utilise TGFβ1. However, I note that figure 2 shows that a polyvalent antiserum, which reacts with all three isoforms, totally abolishes the activity of TGFβ, whereas a specific anti-TGFβ1 antiserum blocks only 86% of this activity. Figure 4 demonstrates that TGFβ1, TGFβ2 and activin are all able to elicit a GM-CSF response, which is the response stated in the specification to be necessary to start the immune cascade which will alleviate the infertility condition. It is clear from Figure 4 that TGFβ2 was as potent as TGFβ1, and that both of these were much more potent than activin; nonetheless activin did have significant activity.

14. I have been provided with a copy in draft form of a statutory declaration by David Sharkey, which I am informed is to be lodged as part of the response to the Office Action. This declaration reports experiments demonstrating that TGFβ1, TGFβ2 and TGFβ3 are all able to induce an increase in GM-CSF release from human cervical keratinocytes, which are cells of the female reproductive tract. I note that the methods used in these experiments are essentially the same as those described on pages 18 and 19 of the present specification.

15. I note that David Sharkey is a PhD. student at the University of Adelaide, and I therefore consider that he is certainly a person of ordinary skill in the art. Using the methods described in the specification he was able to determine that TGFβ3 did have the necessary activity required for the desired effects on fertility. Indeed, TGFβ3 gave a greater stimulation of GM-CSF production by cervical keratinocytes than TGFβ2.

16. I consider that the Sharkey declaration confirms that using the methods described in the specification $TGF\beta_3$ can be identified as suitable for use in the invention, and that these methods would be a matter of routine for a person of ordinary skill in the art.

17. I consider that on the basis of the information provided in the specification, including the experimental results, and on the basis of the general state of the art as it existed at the priority date, it would have been a matter of routine, and would

not have required any inventive effort or undue degree of experimentation, for a person of ordinary skill in the art to have determined that TGFβ3 had the requisite activity to be useful for the purposes of the claimed invention. I similarly consider that such a person of ordinary skill in the art would readily be able to determine whether any other member of the TGFβ superfamily was suitable for these purposes.

18. I therefore consider that the specification provides sufficient information for a person or ordinary skill in the art to identify which subtypes of TGFβ and members of the TGFβ superfamily are suitable for use for the purposes of this invention, and to carry our the invention using the TGFβ molecule thus selected. I consider that the person skilled in the art would have regarded TGFβ1, TGFβ2 and TGFβ3 as being functionally interchangeable for all practical purposes for the purposes of the specification.

19. I note that US Patent No. 539825, cited by the Examiner in an obviousness objection, is stated to encompass all of the isoforms of TGFβ, namely TGFβ1, TGFβ2, TGFβ3 and TGFβ4 (see Column 5 lines 9 to 11). However, only the use of TGFβ1 is exemplified; see example 3, which refers to TGFβ1 (column 9 line 66) and examples 4 and 6, which do not specify the subtype of TGFβ. Notwithstanding this, there is no limitation in the claims to a particular subtype of TGFβ, and the Examiner at page 8 of the Office Action relies on this reference to assert that it would be obvious to utilise administration of *any* subtype of TGFβ together with an antigen, and to combine this with immunisation of the female parent with a paternal leucocyte antigen.

I also note that in the objection to claims 75 and 76, the Examiner asserts that it would be obvious to use modifications or analogues of TGFβ.

20. The Examiner has also objected that the specification does not constitute an enabling disclosure of any antigen other than an MHC Class I antigen on the sperm of the prospective father. I consider that on a fair reading of the specification as a whole, it would be clear to the skilled addressee that it is not necessary that the antigen is actually present on sperm cells; it is merely stated at page 5 lines 31 to 32 that tolerance is elicited "towards male antigens, including paternal MHC Class I antigens". From the passage in

the specification at page 3 lines 24 to 37 it is evident that paternal antigens are present not only in sperm cells but in somatic cells (which are present in ejaculate) and in seminal plasma. At page 8 lines 7 to 9 it is stated that

"the nature of the relevant surface antigens is not entirely clear, but will presumably be those that are particularly antigenic and prominent either on the sperm, or on the conceptus. The most likely candidates are MHC antigens, and more preferably MHC Class I. The most efficient manner of presenting these antigens is in the form that they are naturally present - on any appropriate cell of the intended male parent that expresses them and those cells would include sperm cells and may include leukocytes... This use of cells other than sperm cells will be pertinent where the sperm count of the prospective father is somewhat low. The use of cells other than sperm cells may be preferred where a non-genital route is used... Alternatively the antigens may be presented in purified or semi-purified form, which may or may not be presented on inert or adjuvant carriers...".

While the experimental examples described in the specification disclose only immunisation by intra-uterine infusion with sperm cells, I consider that it would be abundantly clear to a person of ordinary skill in the art that immunisation by other routes, and with immunogens other than sperm cells, could be used. For example, as stated above the specification states that leukocytes may be used; the reference by Chaouat et al cited by the Examiner discloses immunisation with spleen cells, which are predominantly lymphocytes. The specification also refers to the use of purified or semi-purified antigens.

21. Consequently I consider that a person skilled in the art, provided with the information in the specification, would have no difficulty in using antigens other than MHC Class I antigens present on sperm cells, without the need for any inventive effort or an undue degree of experimentation.

22. I am informed that in a previous office action US Patent No. 5395825 by Feinberg et al was cited as the basis for an objection of lack of novelty. The Examiner has now cited this reference in combination with the publications by Clark et al and by Chaouat et al as the basis for objections that the claimed invention is obvious. The

Examiner asserts that Feinberg teaches a method of treating infertility by administering TGFβ together with antigens, such as an ovum, sperm or conceptus, into the reproductive tract of a female to increase the success rate of implantation, and that the TGFβ may be administered before, after or simultaneously with male antigen sperm or antigens from the conceptus.

23. In separate rejections, most of the claims are rejected as being obvious over US Patent No. 5395825 by Feinberg et al, in view of the papers by Clark et al and by Chaouat et al referred to above; claims 66, 67 and 71 are rejected as being obvious over this combination of references, together with references by Harlow et al and by Martin-Villa et al; claims 75 and 76 are alleged to be obvious over this combination of references together with papers by Tuan et al or by Lyons et al; claims 83 to 85 are alleged to be obvious in the light of this combination of references together with Grainger et al; and claim 92 is rejected as being obvious over this combination of references together with Heidenreich et al. I have read and understood the references which have been cited by the Examiner.

24. In fact, the specification by Feinberg refers only to the "competence of a conceptus towards uterine implantation (column 3 line 50) and methods of increasing the success rate of *assisted* reproduction (column 3 lines 66 to 67), and is completely silent regarding antigens of any kind, immunisation of the prospective mother, or immune tolerance. The only mention of any immune factor is at column 4 lines 38 to 47, which states that the invention provides methods of contraception and contragestion comprising administering a TGFβ *antagonist*, such as antibodies to TGFβ, in order to increase the probability that conception will be *prevented.* This is completely opposite to the presently-claimed invention, wherein a TGFβ must be administered to increase the probability that a fertilized ovum will succeed in generating a liveborn offspring.

25. I do not consider that the Feinberg specification provides any actual experimental evidence to suggest that

a) treatment of an ovum or sperm would improve implantation,

b) the prospective mother would be exposed to sperm of a prospective father

together with TGFβ either prior to or after attempted conception, or

c) the TGFβ and antigen of the prospective father should be administered to the prospective mother at different times.

Feinberg suggests that stimulation of fibronectin production by a fertilized ovum by a TGFβ should enhance outgrowth of trophoblast which is essential in the process of implantation, which in reality occurs only *in vivo*. However, Feinberg provides experimental evidence only for treatment of the mother *after* implantation. Since Feinberg provides no demonstration that implantation is enhanced, the proposed biological role of a TGFβ *in vivo* represents untested speculation.

26. The Clark et al reference, of which I am first author, discloses the up-regulation of release of an endogenous TGFβ2-like molecule from CD56+ cells in decidua during the first trimester of pregnancy, ie. after conception and implantation. The decidua is the inner layer of the wall of the uterus, which envelops the embryo and forms part of the placenta. Consequently this is a tissue of maternal origin, not of foetal or paternal origin. This TGFβ2-related molecule is associated with successful, ie. non-aborting, pregnancy in humans. The Clark paper clearly points out that this TGFβ2-related molecule exhibited different biophysical properties compared to those of conventional 25 kD TGFβ2. In that paper, absence of the novel TGFβ2-like molecule, but not absence of a conventional 25 kD TGFβ2, was linked to recurrent spontaneous abortions. It was further shown in a subsequent publication (Clark et al. Biol Reprod. 1995 52 1380-1388) that the novel TGFβ2-related molecule found in the uterus of a successful pregnancy exhibited very different chemical and biological properties compared to conventional TGFβ. A copy of this publication is annexed hereto as Exhibit DAC-3. One would therefore not have expected that administering a conventional TGFβ isoform would prove beneficial in preventing infertility due to spontaneous abortions, since the novel TGFβ2-like molecule, described by Clark et al., is *not* a conventional isoform of TGFβ.

Therefore this reference does not suggest any role of TGFβ prior to implantation, and does not suggest that administration of exogenous TGFβ could be used to alleviate an infertility condition.

27. I do not consider that the finding by Chaouat et al, that administration of a female mouse with spleen cells of a histoincompatible mouse increases fetal viability, suggests that immunisation with paternal MHC class I antigens would be useful for treatment of infertility.

The paper cited by the Examiner in fact shows that prevention of spontaneous abortion in DBA/2-mated CBA/J mice could *not* be prevented by immunising the CBA/J female against the Class I MHC of the DBA/2 male. It was necessary to use spleen cells from BALB/c mice. The BALB/c mouse strain is regarded as having the *same* H-2^d Class I MHC antigen as the DBA/2 strain, and from Kiger et al (J. Immunol. 1985 134 2966-2970), it was subsequently known that minor non-MHC antigens on the BALB/c mouse background were required along with H-2^d-associated MHC antigens. Antibodies are rarely if ever made against minor non-MHC antigens. Data from studies in which women were immunised against their husband's HLA-incompatible blood mononuclear cells (e.g. Mowbray JF, Human Reprod .1988 3 79-82) indicated that an antibody response to MHC was not required for prevention of miscarriages. It was known from Clark et al that immunisation of mice which prevented abortions increased the level of production of the novel TGFβ2-related factor mentioned above, but this should not be taken to indicate that the effect arose from an immune response to the paternal Class I MHC antigens. In fact no published study has shown that immunity to paternal Class I MHC antigens alone can improve the rate of implantation.

28. Feinberg made predictions about the effects of TGFβ on implantation. Clark et al and Chaouat et al were describing phenomena occurring *after implantation had been completed.* Indeed, in other publications, Chaouat et al have reported that immunoprevention of spontaneous abortion in the mouse can occur when the treatment is given as late as 3 days after implantation. It would not have been obvious that TGFβ treatment of preimplantation embryos would have any effect on the phenomena being

investigated by Clark and by Chaouat. Therefore it could not have been obvious to a person of ordinary skill in the field to combine the disclosure of Feinberg with either or both of the Clark disclosures and the Chaouat disclosure. Nor would it have been obvious to such a skilled person to combine the references by Feinberg, Clark et al and Chaouat et al with any of the additional references cited in items 15 to 18 of the Office Action.

I declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like are punishable by prison or fine or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of any application or patent thereon.

DECLARED at Hamilton Ontario this 16th day of December 2002

David A. Clark.

David A. Clark

Before me:

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada.

Declaration under U.S.C.§ Rule 132

IN THE MATTER OF
US Patent Application No. 09/380,327
by ROBERTSON et al.

EXHIBIT DAC-1

This is Exhibit DAC-1 referred to in the Statutory Declaration by David Alexander CLARK

dated 16 Dec 2002

Before me:

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada.

6/12/02 David Clark
DMR-1

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DATE OF BIRTH: April 26, 1943.
St Thomas, Ontario.

EDUCATION:

University of Western Ontario	1960-67
Rotating Intern, St. Paul's Hospital, Vancouver, British Columbia	1967-68
Junior Assistant Resident in Medicine, Victoria Hospital, London, Ontario, and Lemuel Shattuck Hospital, Boston, Mass., USA	1968-69
Senior Assistant Resident in Medicine, University of Western Ontario, Department of Medicine	1969-70
Clinical Research Fellow in Oncology and Immunology, Tuft's New England Medical Center, Boston, Mass., USA	1970-72

	Member Eastern Cooperative Oncology Group	1970-72
	Graduate Student, Institute of Medical Science, University of Toronto, and Ontario Cancer Institute, Toronto, Canada	1972-76
DEGREES AND DIPLOMAS:	M.D., University of Western Ontario (Gold Medalist, summa cum laude)	1967
	L.M.C.C. (Licentiate Medical Council of Canada)	1968
	Diplomate American Board of Internal Medicine, Boston, Mass., USA	1972
	Fellow, Royal College of Physicians and Surgeons of Canada (Internal Medicine)	1974
	Ph.D. (Thesis: Regulation of Cytotoxic T lymphocytes) R.G. Miller, Supervisor, University of Toronto	1977
	Fellow of the Royal College of Physicians (Edinburgh, UK)	2001
HONOURS:	Ontario Government Scholarship (Ontario Scholar)	1961
	Khaki University and YMCA Scholarship	1961
	University of Western Ontario Board of Governor's Scholarship	1962
	Mathematics Class of 1949 Prize	1962
	J.P. Campbell Scholarship in Physiology	1964
	Mosby Histology Prize	1964

	CIBA Award	1965
	Mosby Award	1965
	Medical Alumni Gold Medal	1967
	Eccles Scholarship, Poulenc Award & Gold Medal	1967
	MRC Fellowship	1972-77
	MRC Scholarship	1980-84
	MRC Scientist Award	1984-88
	Fellow, Royal Society of Medicine (London,UK)	1992-
	President-elect, American Society for Reproductive Immunology	1995-96
	President, ASRI	1996-98
	President-elect, International Society for Immunology of Reproduction, (ISIR)	1995-98
	President, ISIR	1998-2001
	President d'Honneur, ISIR	2001-
	Gold Medal of the ISIR	2001
	Fellow, Royal College of Physicians (UK)	2002
CURRENT STATUS:	Associate Member Obstetrics & Gynecology	11-1984-
	Associate Member Molecular Medicine and Pathology	2-1993-
	Tenured regular appointment - Medicine	01-1989-02
	Emeritus Professor of Medicine, (+ Molecular Medicine & Pathol. + Ob-Gyn. + Graduate Program in Health Sciences) McMaster University	2002-

	Professor, Institute of Medical Sciences, Univ. Toronto Faculty of Graduate Studies	2002-

PROFESSIONAL ORGANIZATIONS:

Vice President ASRI	1986-87
Councillor ASRI - elected	1994-
Councillor ISIR - elected	1986-89
Councillor ISIR - Re-elected	1989-92
Presidential Reappointment to Council	1992-95
President-Elect ASRI	1995-6
President - ASRI	1996-98
Past-President – ASRI	1998-00
President-Elect ISIR	1995-8
President - ISIR	1998-2001
Past-President – ISIR	2001-

EMPLOYMENT HISTORY:

Assistant Professor of Medicine, McMaster University, (Tenured)	1976-82
Associate Professor of Medicine, McMaster University, Hamilton	1982-86
(secondary i.e. cross appointment) Obstetrics/Gynecology)	1982-86
Professor of Medicine, McMaster University, Tenured	1986-
(secondary i.e. cross appointment Obstetrics/Gynecology	1986-
(secondary appointment) Pathology	1993-
Consultant in Medical Oncology, Ontario Cancer Foundation, Hamilton Clinic	1977-
Member, Graduate School Faculty, McMaster University, Hamilton	1977-
Affiliate Staff, Hamilton Civic Hospitals, Hamilton	1976-
Associate Staff, McMaster University Medical Centre, Hamilton	1977-78

Active Staff, McMaster University Medical Centre, Hamilton Health Sciences Corporation, Hamilton	1978-
Associate Member, Reproductive Biology Program	7-1984-
WHO Advisor	11-12-1988
Member, Board of Comprehensive Examination Chairs Graduate Program Faculty of Health Sciences	1998-2004
Chair, Palliative Care Committee McMaster Unversity Medical Center	1980-1990
Member, Research Ethics Board, HHSC	1999-
Member, Pharmacy & Therapy Committee, Hamilton Health Sciences	2001-
Affiliate Scientist, Organ Transplant, Dept. Cell Molec. Biol., Univ. Health Network, U of Toronto	2001-

SCHOLARLY AND PROFESSIONAL ACTIVITIES:

Editorial Boards:

Editorial Board, Journal of Reproductive Immunology	1985-1992
Editorial Board, American Journal of Reproductive Immunology	1985-
Associate Editor, Journal of Immunology, Section of Clinical Immunology and Immunopathology	1985-1989
Editorial Board, Regional Immunology	1987-1992
American Journal of Reproductive Immunology	1984-

	Associate Editor for North America, American Journal of Reproductive Immunology	1992-
Grant and Personnel Committees:	Member - Applications Committee – Banting Research Foundation	1979-
	Member - MRC Fellowship Panel	1983-1985
	Scientific Officer, MRC Grants Panel for Immunology and Transplantation	1985-1990
	MRC Group Site Review Team	1994
	Member-MRC - PMAC Grants Panel	1995-1998
	-MRC - Senior/Distinguished Scientist Awards Panel	1995-1998
	HED NIH Study Section member	2001-
Executive Position:	6th International Congress of Immunology, Toronto, Canada (Chair, Organizing Committee)	1986
	NCIC Lung Cancer Clinical Trials Committees (2)	1982-86
	Chairman of Local Organizing Committee (3rd International Congress of Reproductive Immunology)	1983-86
	Member of Scientific Committee, Immunology of Normal & Diabetic Pregnancy (Erice, Sicily)	1990
	Program Committee (ASIR) Ethics Committee (ASIR) (Chair of Statistical Analysis Subcommittee)	1990-
	Associate Editor for North America, American Journal of Reprod. Immunol.	1992-
	Program Committee 6th Int. Congress Reprod. Immunol.(Washington, 1995)	1992-95
	Program Committee 1993 ASIR-AAI meeting	

	(Denver)	1992-93
	Program Committee Annual ASRI meetings	1994-2002
	NIH/NICHD Scientific Study Group, Bethesda, Md. USA (ad hoc)	1992-
	President-elect, Amer. Soc. Reprod. Immunol.	1995-96
	President, ASIR	1996-98
	Program Committee - 4th Banff Conference	1996
	President-Elect, International Society Reprod. Immunol.	1995-98
	NIAID/NIH Task Force on Immunology (Co-Chair)	1997-98
	President ISIR	1998-2001
	Member, International Scientific Program Committee, 12th International Congress of Immunology (Montreal, 2004)	2001-2004
	Member, NIH HED Study Section	2001-
	Editorial Board, Journal of Assisted Reprod. and Genetics	2001-
	Safety Monitor, US FDA Immunotherapy Study	2002-
Journal Referee:	Journal of Immunology	1979-
	Regional Immunology	1988-
	American Journal of Reproductive Immunology	1983-
	Journal of Reproductive Immunology	1983-
	Journal of Reproduction and Fertility	1984-
	Fertility and Sterility	1986-
	Immunopharmacology	1986-
	American Journal of Obstetrics & Gynaecology	1988-
	Immunology Letters	1984-
	Reproduction, Fertility & Development	1989-
	Journal of Clinical Immunology	1990-
	New England Journal of Medicine	1990-

Biology of Reproduction	1990-
Developmental Biology	1990
Periodicum Biologicum	1990-
Lancet	1992-
Human Reproduction Journals	1992-
Cellular Immunology	1980-
Immunology (BSI, UK)	1999-
Clinical & Experimental Immunol	2001-
Proc. Soc. Exp. Biol. Med.	1983-

External Grant Reviews: World Health Organization
MRC New Zealand
National Cancer Institute of Canada
MRC/CIHR Canada
National Institutes of Health (USA)
Ministry of Health (Ontario, Manitoba, Alberta, & BC)

AREAS OF INTEREST: Reproductive Medicine and Solid Tumor Systemic Therapy:
Consulting
Teaching
Research

COURSES TAUGHT:

Discipline Coordinator and Large Group Leader - Immunology MD Program	1982-86
Advance Immunobiology (715) (given alternate years to 739, average 2-4 sessions)	1978-92
Seminar Leader and Course Module Co-ordinator	1992-97
Module II Coordinator/Leader MS715A (Yearly)	1997-2001
General Pathology (729) Seminar Leader	1979-
Immunogenetics (739) Seminar Leader (given alternate years to 715, average 2-3 sessions) (Currently requested in 2001)	1983-97
Reproductive Immunology (777) Seminar Leader (given when scheduled, average 2-4 sessions)	1979-

	Unit 2 Tutor and Unit 3 Tutor	1996,1998,2000 2001
	4ii3 Undergraduate Immunology (Lecturer & Examiner)	1997-

SUPERVISORSHIPS:

Master:	R. Slapsys (complete)	1983
	J. Brierley (complete)	1986
	S. Daya (complete)	1986
	F. Merali (complete)	1993
	K. Yu (to begin May 2001)	
Doctoral:	R. Slapsys (complete)	1987
	K. Wright (complete)	1987
	O. Porat (complete)	1991
	A. Cadavid (complete 1998)	1998
	M. Knackstedt (In progress)	1999
Post-Doctoral:	J. Kreck (complete)	1983
	P. Nagarkatti (complete)	1983
	R. Lea (complete)	1988-91
	H. Hirte (complete)	1990
	E. Muzikova (complete)	1993
	S. Wang (complete)	1993
	P. Arck (complete)	1993-96
MD Research Electives:		
	J. Neiss (complete)	1998
	A. Keil (complete)	2000
Professional:	MD Program (OPD Oncology Rotation in Ambulatory Care, MUMC/HSC)	1987-
	CTU (Medicine Physician)	1988,1990
	Immunotherapy Program for recurrent Pregnancy failure couples (with Dr. Salim Daya)	1988-

Supervisory Committees:	M. Evelegh	(complete)	1980
	M. Chan	(")	1982
	S. Gee	(")	1979
	K. Rosenthal	(")	1979
	K. Wright	(")	1980

	P. Ernst	(")	1986
	J. Toles	(")	1986
	M. Romi	(")	1991
	F. Merali	(")	1993
	S. Gyorffy		1994 (resigned)
	A. Cadavid	(")	1998
Student Advisor	F. Dossa	(complete)	1999-02
Student Advisor	A. Duggal	(complete)	1998-01
Student Advisor	C. Chin		2001-
Comp Exam	S. Sweet		1996
Ph.D. Exam	H. Zhong		1997
MSc Defense	L.A. Cohen		1997
Comp Exam	S. Carter		1998
Comp Exam	J. Preston		1999
Comp Exam	N. Sharan		2000
Comp Exam	G. Trentin		2000
Comp Exam	D. Dowlatshahi		2000
Comp Exam	B. Gajewska		2000
Comp Exam	S. Ritz		2000
Comp Exam	P. Margettes		2001
Comp Exam	P. Nair		2002
Comp Exam	D. Alvarez		2002
PhD Transfer Exam.	R. Khadaroo (U of Toronto)		2002

Ph.D. Examiner (written and oral 'viva'): L. Flynn, University College, Dublin, Ireland 7/2000

CURRENT RESEARCH FUNDING:

Canadian Institutes of Health Research (CIHR)
"Immunopathogenesis of viral hepatitis"
(with G.A. Levy), $186,417/yr 2000-2005

Bayer-Blood Partnership "Mechanism of transfusion-related immunomodulation: prevention and exploitation" (with M. Blajchman) $ 61,500/yr 2001-2003
[this grant received highest peer-reviewers' score in Canada]

Alexander von Humboldt Foundation 2002
Eu 50,000/yr research award.

ASSIGNED INTELLECTUAL PROPERTY (INVENTIONS):

1. Antagonism of procoagulant as a mechanism of prevention of early pregnancy failure. 2/1999
   Clark DA

2. Novel prothrombin cleavage as a basis for diagnosis and treatment in human pregnancy. 5/1999
Clark DA, Weitz J, Hirsh J

3. OX-2 ligands: detection of OX-2 receptor. 5/2000
Clark DA

4. Anti-OX-2 antibody blocks stimulation of tumor growth and metastases by transfused allogeneic white blood cells and allows white cells to stimulate anti-tumor effect in vivo.
Clark DA 11/2000

5. Release of a soluble bioactive OX-2 (CD200) from leukocytes in vitro.
Clark DA 5/2001

6. Immunomodulation of MD1 to prevent fetal loss syndrome, pre-eclampsia, and other LPS-dependent pathology.
Clark DA 7/2001

PATENTS:

1. **Title**: Methods of Modulating Immune Coagulation
International Application #: PCT/CA98/00475
International Application Date: May 15th, 1998
Filing Date: November 15th, 1999
Inventor(s): Levy, Gary A; Clark, David A.
Patent awarded: US 2001 #6403089, Australia 2001

2. **Title:** Methods and Compositions for Immunomodulation
International Application #: PCT/CA98/01038
International Application Date: November 6, 1998
Filing Date: January 21, 2000
CIP: Methods for modulating tumor growth 27/7/2001
CIP: Methods and compositions for modulating fertility 23/8/2001
Inventor(s): Gorczynski, Reginald M.; Clark, David A.

3. **Title:** Methods and Composition for Immunoregulation
U.S. Provisional: 60/189985
Filing Date: May 7, 2000
PCT/CA01/00346 16/3/2001
Inventor(s): Gorczynski, Reginald M & Clark, David A.

CO-INVESTIGATOR ON CLINICAL TRIALS:

1: Zaledronate 11
2: Zaledronate 10
3: Petacc/Tomudex vs 5FU (Astra Zeneca)
4: CPT 11 vs Oral Topo 5 days vs Oral Topo 21 days
2nd line management; colon cancer

5: NCIC CO 14
6. PA-3(Principal-Investigator)

LIFETIME PUBLICATIONS:

EDITED REVIEWS & BOOK CHAPTERS & OTHER CONTRIBUTIONS

1. Clark, D.A. 1966. Mechanisms of mood. In: University of Western Ontario Medical Journal 36(3):74-84.

2. Clark, D.A. and L. Nathanson. 1972. Cellular immunity in malignant melanoma. In: The Pigment Cell, Vol.I. Edited by V. Riley. Karger Basel, pp 350-359.

3. Clark, D.A. and L. Nathanson. 1972. Patterns of cellular immunity in malignant melanoma. In: Recent Results of Cancer Research. Edited by G. Mathe, Springer-Verlag, pp. 67-78.

4. Nathanson, L. and D.A. Clark. 1973. *In vitro* assay of cell-mediated immunity in BCG therapy of malignant melanoma: a preliminary report. National Cancer Inst. Monogr. 39:221-224.

5. Nathanson, L., Necheles, T.F, Clark, D.A., Silverman, E. and D. Whitten. 1975. HLA-5 masking activity in malignant melanoma. Behring Inst. Mitt. 56:97-103.

6. Phillips, R.A., Clark, D.A., Schilling, R.M. and R.G. Miller. 1975. Quantitation and characterization of effector cells and their progenitors. In: Proc. International Cancer Congress, Florence, Italy. 1974. American Elsevier Publishing Co., New York. Vol.I, pp. 264-269.

7. Miller, R.G., Clark, D.A., Harley, E. and R.A. Phillips. 1975. Quantitation of killer T cell-target interaction: suppressor cells at the effector stage. In: Suppressor Cells in Immunity. International Symposium, May 1975. Eds. S.K. Singhal and N.R. St. C. Sinclair. University of Western Ontario, London, Canada, pp. 50-60.

8. Gee, S.R., Chan, M.A., Clark, D.A. and W.E. Rawls. 1980. Genetically acquired resistance to fatal Pichinde virus infection in the Syrian hamster. In: Genetic Control of Resistance to Infection and Malignancy. An International Symposium of the Canadian Society for Immunology. Eds. E. Skamene, P.A. Konghavn and M. Landy. Academic Press, New York, pp. 345-352.

9. Gee, S.R., Chan, M.A., Clark, D.A. and W.E. Rawls. 1980. Susceptibility to fatal Pichinde virus infection in the Syrian hamster. Proc. of Symposium on Hamster Immune Responsiveness and Experimental Modes of Infectious and Oncologic Diseases, June 3-5, 1980. Eds: J.W. Streilein, D. Hart, J. Stein-Streilein, W. Duncan and R.E. Billingham, pp. 327-328.

10. Clark, D.A. 1982. Impairment of host immunity and survival of the fetus. In: Immunobiology of Transplantation, Cancer, and Pregnancy. P.K. Ray, Editor, Pergamon Press, Inc. New York, pp. 278-304.

11. Clark, D.A., Gauldie, J., Sweeney, G. and S. Safe. 1982. Suppression of immune defences by halogenated aromatic hydrocarbons. In: Proc. Second Annual Ontario Ministry of Environment Technology Transfer Conference, November 24, 1981.

12. Clark, D.A., Slapsys, R.M. and M.R. McDermott. 1982. Active localized suppression of killer T cells by suppressor cells in the genital tract of allopregnant mice. In: Immunology of Reproduction. Eds. K. Bratanov, V.H. Vulchanov, V. Dikov, B. Somlev, R. Georgieva and A. Tornyov. Bulgarian Acad. Sci. Press, pp. 418-421.

13. Clark, D.A. 1982. The Promise of RI. A review of the American Journal of Reproductive Immunology. Nature, 299:504.

14. Clark, D.A., Slapsys, R., Croy, B.A., Rossant, J. and M. McDermott. 1983. Regulation of cytotoxic T cells in pregnant mice. In: Immunology of Reproduction. Eds. T. Wegmann and T.J. Gill. III. Oxford Press, New York, pp. 342-361.

15. Clark, D.A., Sweeney, G. and J. Gauldie. 1983. Suppression of immune defences by halogenated aromatic hydrocarbons. In: Proc. Technology Transfer Conference No. 4, Nov. 29-30, 1983, Ontario Ministry of the Environment, pp. 301-308.

16. Clark, D.A. 1984. Role of hormonal immunoregulation in reproduction. In: Pituitary Function and Immunity. Ed: I. Berczi. CRC Press, Inc., Florida, pp. 261-272.

17. Clark, D.A. 1984. Immunoregulatory mechanisms in pregnancy of animals. In: Reproductive Immunology. Eds: S. Isojima and W.D. Billington. Elsevier Science Publishers, New York, pp. 259-262.

18. Clark, D.A., Gauldie, J. and G. Sweeney.1984. Dose response, time-course and mechanism for suppression of cytotoxic cell generation by 2,3,7,8-tetrachlorodibenzo (P) dioxin. In: Mechanisms for Biological Effects of Dioxins. Cold Spring Harbour, Banbury Report 18:421-434.

19. Clark, D.A. and R. Slapsys. 1985. Immunoregulatory mechanisms in the uterus and survival of the fetus. In: Contributions to Obstetrics and Gynecology, P.J. Keller, Zurich. Karger Basel, 14:44-53.

20. Clark, D.A. 1985. Maternofetal relations: a minireview. Immunol. Letters 9:239-247.

21. Clark, D.A. 1985. Maternal immune response to the fetus. In: Current Trends in Immunology of Human Reproduction. EOS - Revista di Immunologia ed Immunofarmacologia, eds: F. Dondero and S. Shulman. Vol. VI, pp. 114-117.

22. Wright, K.E., Clark, D.A. and W.E. Rawls. 1985. Pichinde virus infection and differences in lymphocyte responsiveness to lymphokines in two inbred strains of Syrian hamster. In: Genetic Control of Host Resistance to Infection and Malignancy pp. 203-209.

23. Clark, D.A. 1985. Oxford Review of Reprod. Biol. Vol. 6, 1984. J. Reprod. Immunol. A review. Vol. 8, pp. 262-264.

24. Clark, D.A. 1985. Survey of Drug Research in Immunological Disease. Vol. 4. Noncondensed Aromatic derivatives. Part III. Vol. 5. Noncondensed Aromatic Derivatives. Part IV. Ed: V. St. Georgiev. Karger, Basel. Book Review. J.A.C.S. 107:25;7800-7801.

25. Clark, D.A. 1985. Prostaglandin and immunoregulation during pregnancy. Editorial Review. Amer. J. Reprod. Immunol. Microbiol. 9:111-112.

26. Clark, D.A., Sweeney, G.D. and J. Gauldie. 1985. Quantitative assessment of immunotoxicity of haloaromatic hydrocarbons. Key role of the thymus gland in susceptibility to dioxin and implications for definition of "no effect" exposure levels. Proc. Technology Transfer, Conf #6, Dec. 11-12, 1985. Ontario Ministry of the Environment. ISSN 0-825-491.

27. Clark, D.A. and T.G. Wegmann. 1986. Reproductive Immunology. In: Immunology in Canada. Immunology Today Suppl. May, 1986. pp. 28-29.

28. Clark, D.A., Damji, N., Chaput, A., Daya, S., Rosenthal, K.L. and J. Brierley. 1986. Decidua-associated suppressor cells and suppressor factors regulating interleukin 2: their role in the survival of the "fetal allograft". In: Progress in Immunology VI (Eds. B. Cinader, R.G. Miller). Academic Press Inc. pp. 1089-1099.

29. Clark, D.A., Brierley, J., Slapsys, R., Daya, S., Damji, N., Chaput, A. and K. Rosenthal. 1986. Trophoblast-dependent and trophoblast-independent suppressor cells of maternal origin in murine and human decidua. In: Reproductive Immunology, 1986. (Eds. Clark, D.A. and B.A. Croy). Elsevier, Amsterdam pp. 219-226.

30. Daya, S., Lee, S., Underwood, J., Mowbray, J., Craft, I. and D.A. Clark. 1986. Prediction of outcome following transfer of *in vitro* fertilized human embryos by measurement of embryo-associated suppressor factor. 3rd ICRI. In: Reproductive Immunology, 1986. (Eds. Clark, D.A. and B.A. Croy), Elsevier, Amsterdam pp. 277-285.

31. Clark, D.A., Chaput, A., Slapsys, R.M., Brierley, J., Daya, S. and R. Allardyce. 1987. Suppressor cells in the uterus. In: Immunoregulation and Fetal Survival. Eds. T. Wegmann and J. Gill. Oxford Univ. Press, New York, pp. 63-77.

32. Clark, D.A., Chaput, A., Slapsys, R., Brierley, J., Daya, S., Rosenthal, K. and G. Chaouat. 1987. Local intrauterine suppressor cells and suppressor factors in survival of the fetal

allograft. In: Reproductive Immunology: materno-fetal relationship. INSERM Colloque. Vol. 154, Paris, pp. 77-88.

33. Croy, B.A., Crepeau, M., Yamashiro, S. and D.A. Clark. 1987. Further studies on the transfer of Mus caroli embryos to immunodeficient Mus musculus. In: INSERM Colloque. Vol. 154, Paris, France, pp. 101-112.

34. Chaouat, G., Lankar, D., Kolb, J.P. and D.A. Clark. 1987. 2 modeles d'avortements d'origine immunitaire chez la souris de laboratoire: mecanismes abortifs, modalites et mecanismes de traitement par immunisation contre un male, relie ou non relie, suivant les differences antigeniques pere-mere. In: INSERM Colloque, Vol. 154, (Chaouat G, ed), Paris, France, pp. 243-254.

35. Clark, D.A. 1987. Current concepts of immunoregulation in implantation. In: Implantation, (Chapman M.G. and J.G. Grudzinskas, eds), Springer-Verlag, London, pp. 163-175.

36. Clark, D.A. 1987. On pre-eclampsia and leukocytes in human decidua. Editorial on Khong's paper, Amer. J. Reprod. Immunol. Microbiol. 15:9-11.

37. Clark, D.A. 1987. Immunology and Birth Defects: Introduction to AJRIM special issue. Amer. J. Reprod. Immunol. Microbiol. 15:131-132.

38. Clark, D.A., Goddard, G. and G.D. Sweeney. 1987. Assessment of Toxicity of Digested and Inhaled Haloaromatic Hydrocarbons. MOE Paper. In: Proc. 1987 Tech. Transfer Conf. pp. 21-36.

39. Clark, D.A. 1988. Pregnancy Immunology. Year Book of Science. McGraw-Hill, pp.340-344.

40. Clark, D.A. 1988. Host immunoregulatory mechanisms and the success of the conceptus fertilized *in vivo* and *in vitro*. In: Early Pregnancy Loss: Mechanisms and Treatment (Beard R.W. and F. Sharp, eds) Peacock Press. Ashton-Under-Lyne, U.K., pp. 215-227.

41. Chaouat, G., Clark, D.A. and T.G. Wegmann. 1988. Genetic aspects of the CBA x DBA/2 and B10 x B10.A models of murine pregnancy failure and its prevention by lymphocyte immunisation. In: Early Pregnancy Loss: Mechanisms and Treatment (Beard R.W. and F. Sharp, eds) Peacock Press, Ashton-Under-Lyne. U.K. pp.89-102.

42. Clark, D.A. and S. Daya. 1988. Macrophages and other migratory cells in endometrium: relevance to endometrial bleeding. WHO Symposium on "Contraception and Mechanisms of Endometrial Bleeding", (D'Arcangues C., Fraser I., Newton J. and V. Odlind, eds), Cambridge University Press, pp. 363-382.

43. Clark, D.A., Mowbray, J., Underwood, J. and M. Michel. 1988. On granulated cells in human endometrium. Letter to editor. Amer. J. Immunol. Microbiol. 14:144-146.

44. Clark, D.A. and S. Daya. 1988. Immunosuppressive activity in IVF supernatants. Letter to Editor Amer. J. Reprod. Immunol. Microbiol. 17:156-157.

45. Clark, D.A. 1989. The Immunology of Recurrent Abortion. Chapter 2. Adv. in Obstetrics & Gynecology, (Bonnar J., ed) Vol. 16, Churchill Livingstone, London, pp. 25-42.

46. Lea, R.G. and D.A. Clark. 1989. The immune function of the endometrium. In: Bailliere's Clinical Obstetrics & Gynecology "Dysfunctional Uterine Bleeding and Menorrhagia", (J. O'Drife, ed), Vol.3, pp.293-313.

47. Clark, D.A. and G. Chaouat. 1989. Determinants of embryo survival in the peri- and post-implantation period. Serono Symposia: Blastocyst Implantation, Yoshinoga, K. ed., Adams, Boston, pp.171-178.

48. Clark, D.A. 1989. Cytokines and Pregnancy. In: Current Opinion in Immunology, (Roitt I., ed), Vol.1, Alden Press, London, pp. 1148-1152.

49. Mogil, R., Guilbert, L., Clark, D.A. and T.G. Wegmann. 1989. Immunological cross talk between fetal placental cells and T lymphocytes. Cellular Basis of Immune Modulation, Alan R. Liss, pp.171-174.

50. Clark, D.A. 1989. The origin and role determining success and failure in the post-implantation period. In: Cellular Signals Controlling Uterine Function. L.A. Lavia ed., Plenum pp.145-155.

51. Clark, D.A., Banwatt, D.K., Manuel, J., Fulop, G. and B.A. Croy. 1989. SCID mice in reproductive biology. In: "Current Topics in Microbiology and Immunology" (M.J. Bosma, R.A. Phillips & W. Schuller, eds), Vol. 152, Springer-Verlag, Heidelberg 1989, pp.227-234.

52. Feld, R., Rapp, E., Pater, J.L., Willan, A., Cormier, Y., Murray, N., Evans, W. K., Hodson, D.I., Clark, D.A., Arnold, A.M., Ayoub, J.I., Wilson, K.S., Latreille, J., Wierzbicki, R.F. and D.P. Hill. 1989. Prolonged survival in patients with advanced non-small cell lung cancer with combination chemotherapy - Report of a Canadian multicentre randomized trial. In: Treatment and Prevention of Small Cell Lung Cancer, (Gralla, R.J. & Einhorn, L.H., eds), International Congress and Symposium Series 150, pp.83-93.

53. Chaouat, G., Menu, E., Kinsky, R., Dy, M., Minkowski, M., Dolage, J., Thang, M.N., Clark, D.A. and T.G. Wegmann. 1989. Lymphokines and non-specific cellular lytic effectors at the feto-maternal interface: effect on placental size and survival. In: Reproductive Immunology 1989, DW Billington ed, Elsevier, Amsterdam 1989.

54. Clark, D.A. 1990. Decidua-placenta immunologic interactions. CRC Press. In: Immunology of the Fetus (Chaouat G., ed). CRC Press 13, Boca Raton, Fl., pp. 162-170.

55. Daya, S. and D.A. Clark. 1990. Immunoregulation at the materno-fetal interface: the role of suppressor cells in preventing fetal allograft rejection. In: Immunology and Allergy Clinic of North America. (N. Gleicher, ed) Vol.10, W.B. Saunders, Philadelphia, pp.49-64.

56. Clark, D.A. 1990. Lymphokines and cytokines affecting reproductive outcome. In: Immunobiology of Normal and Diabetic Pregnancy. (U. di Mario, ed) John Wiley & Sons, Chichester, Chapter 5, pp.79-90.

57. Clark, D.A. 1990. Animal models of normal and aborting pregnancy. In: Immunobiology of Normal and Diabetic Pregnancy. (U. di Mario, ed) John Wiley & Sons, Chichester, Chapter 2, pp.23-37.

58. Clark, D.A. 1990. Are there immune abortions. FORUM 1990. Res. Immunol. (Inst. Pasteur) 141:202-207.

59. Clark, D.A. 1990. Paraimmunology in the decidua? Am. J. Reprod. Immunol. 24:37-39.

60. Clark, D.A. and S. Daya. 1990. Recurrent abortion: is it treatable? CFAS Controversies in Reproductive Endocrinology: an Update for the Clinician. Serono Symposia, Oct/90.

61. Clark, D.A. and S. Daya. 1990. Macrophages and other migratory cells in endometrium: relevance to endometrial bleeding. In: WHO Symposium Contraception and Mechanisms of Endometrial Bleeding, (D'Arcangues C., Fraser I., Newton J. & Odlind V., eds), Cambridge University Press, Cambridge, pp.363-382.

62. Clark, D.A. 1990. Commentary on the FORUM. FORUM 1990. Res. Immunol. (Inst. Pasteur) 141:218-226.

63. Chaouat, G., Menu, E., Hoffmann, M., Dy, M., Minkowski, M., Clark, D.A. and T.G. Wegmann. 1990. Lymphokines at the feto-maternal interface affect fetal size and survival. In: Placental Communications: Biochemical, Morphological and Cellular Aspects. L. Cedard, E.Alsat, J. Chalier, G. Chaouat and A. Malassine, eds. Colloque INSERM, John Libbey Eurotext, Paris, Vol. 199, pp.133.

64. Lea, R.G., and Clark, D.A. 1991. Macrophages and migratory cells in endometrium relevant to implantation. (Seppalla W., ed). In: Bailliers Clinic. Obstet. Gynecology Chapter 5. pp.25-59.

65. Clark, D.A., Head, J.R., Drake, B., Fulop, G., Brierley, J., Manuel, J., Banwatt, D. and G. Chaouat. 1991. Role of a factor related to transforming growth factor β2 in successful pregnancy. In: Molecular Immunology of the Feto-maternal Interface. (Gill. T. & T.G. Wegmann, eds). Oxford University Press pp 294-311.

66. Chaouat, G., Menu, E., Szekeres-Bartho, J., Rebut-Bonneton, C., Bustany, P., Kinsky, R., Clark, D.A. and T.G. Wegmann. 1991. Lymphokines, steroids, placental factors and trophoblast intrinsic resistance to immune cell mediated lysis are involved in pregnancy

success or immunologically mediated pregnancy failure. In: Molecular Immunology of the Feto-maternal Interface (Gill T. and T.G. Wegmann, eds). Oxford Univ. Press pp 277-293.

67. Clark, D.A. and C. Blair (eds). 1991. NIHCD Conference on Materno-feto Placental Interactions. J. Reprod. Fert. 91:232-244.

68. Coulam, C. and Clark, D.A.,1991. Report of ASRI Ethics Committee on Treatment of Recurrent Miscarriage. Am. J. Reproductive Immunology. 26:93-95.

69. Clark, D.A., Lea, R.G., Denburg, J., Banwatt, D., Manuel, J., Damji, N., Underwood, J., Michel, M., Mowbray, J., Daya, S. and G. Chaouat. 1991. TGF-β related suppressor factor in mammalian pregnancy and in recurrent unexplained abortion. In: Proc. INSERM Colloque "Materno-fetal Relationship: Molecular and Cellular Biology", (Chaouat G. ed), John Libbey Eurotext, Paris pp. 171-179.

70. Clark, D.A. and R.W. Beard. 1991. A morphological and immunological study of human placental bed biopsies in miscarriage. Author's Reply. Brit. J. Obstet. Gynecol. 98:611.

71. Clark, D.A., Lea, R.G., Podor, T., Daya, S., Banwatt, D., and Harley, C. 1991. Cytokines determining the success or failure of pregnancy. Ann. NY Acad. Sci. 626:524-536.

72. Daya, S. and D.A. Clark. 1991. Immunology of habitual abortion. SOGC Symposium, June 1991.

73. Lea, R.G., Harper, J., Banwatt, D., Manuel, J., Daya, S., Porat, O., Craft, I. and D.A. Clark. 1991. Is there an early embryo associated suppressor factor? In: Proc.INSERM Colloque "Materno-fetal Relationship: Molecular and Cellular Biology", (Chaouat G., ed), John Libbey Eurotext, Paris pp 161-168.

74. Daya, S. and D.A. Clark. 1992. Alloimmunity in normal pregnancy and pregnancy loss. In: Principles of Medical Therapy in Pregnancy, 2nd Edition (N. Gleicher, ed), Appleton & Lange, Norwalk pp 407-413.

75. Clark, D.A. 1992. Animal Models of Early Pregnancy Loss. In: Immunologic Obstetrics, (Coulam C., Faulk W.P. & J. McIntyre, eds), W.W. Norton, New York pp 428-436.

76. Coulam, C., Clark, D.A., and A.E. Beer. 1992. Report from the Ethics Committee for Immunotherapy. AJRI 28:3-5.

77. Daya, S. and D.A. Clark. 1992. *In vitro* fertilization in immunological infertility. Ann. Acad. Med., Singapore. Vol 21:4 pp 525-532.

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80. Clark, D.A. 1992. Anti-sperm antibodies: do they or don't they? SCOG Symposium. June 1992.

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82. Clark, D.A. 1993. Cytokines and uterine bleeding. In: Steroid Hormones and Uterine Bleeding (N. Alexander, C.D. Arcangues, eds). AAAS Press. Washington, pp. 263-275.

83. Clark, D.A. 1993. Paraimmunology & receptors on decidual T-cells: conflict or confirmation? Am. J. Reprod. Immunol. 29:35-38.

84. Clark, D.A. and S. Daya. 1993. Immunological therapies of recurrent pregnancy loss. In: Textbook of Gynecology, (Copeland L.J. ed), W.B. Saunders, Philadelphia pp 231-241.

85. D.A. Clark. 1994. Endometrial molecular and cellular determinants of fertility (and sterility). In: Progress in Infertility. (Behrman, S.J., Patton, G.W., Holtz, G. eds.) Little, Brown & Co. pp.37-44.

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172. Keil A, Yu K, Manuel J, Levy GA, Gorczynski RM, Clark DA. 2001. The 'tolerance-promoting' molecule OX-2 is expressed in fetal trophoblast cells that cocoon the 'fetal allografts' and may prevent pregnancy loss caused by activation of fgl2 prothrombinase. Amer. J. Reprod. Immunol. 45;343.

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173. Clark DA, Gorczynski RM, Levy GA. 2002. One signal to abort, or 3 for safety ? Definition of the molecular matrix- the importance of danger and its immunotherapeutic antithesis. Amer. J. Reprod. Immunol. 47:356

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PRESENTATIONS AT MEETINGS:

Invited:	American Society for Immunology of Reproduction, Park City, Utah (Keynote Speaker)	06-1983
	University of Liverpool, Department of Immunology (Speaker) (Evidence for Immune Rejection of the Fetus)	04-1984
	King's College Cambridge, British Society of Immunology (Lecturer)(Suppressor Cells and Success of the Fetal Allograft)	04-1984
	Institut Curie, Paris, France,	

(Speaker)(Pregnancy Immunology)	04-1984
Cold Spring Harbor Symposium "Biological Mechanisms of Dioxin Action" (given by Dr. Sweeney due to previous commitment to speak in England)	06-1984
Canadian Society for Immunology, Symposium on Immunology of Reproduction (Symposium Speaker)	06-1984
Gordon Conference on Mammalian Genital Tract (Speaker) (Pregnancy Immunology)	07-1984
RES Society Minisymposium on Immunotoxicology (Speaker)	10-1984
International Congress on Reproductive Immunology, Tel Aviv (Symposium Speaker)	10-1984
Minisymposium of Reproductive Immunology, Paris, France (Speaker)	10-1984
6th International Symposium on Immunology of Reproduction, Varna (Speaker)	05-1985
Anatomical Society of Great Britain, University of Southampton, U.K. (Pregnancy Immunology)	01-1986
Inserm Colloque, Paris, France, Immunology of Reproduction (Speaker and Member of Scientific Committee)	12-1986
Implantation Meeting, Guy's Hospital, London (Speaker) (Current concepts of immunoregulation of implantation)(Speaker)	04-1987
Philadelphia, Spring Symposium in Clinical Applications of Reproductive Immunology (Immunological aspects of pregnancy and IVF failure and their treatment)	

(Clinical approaches to infertility and recurrent abortion)(Speaker) 05-1987

Gordon Conference
"Are immunosuppressors and growth stimulating factors the same?" (Speaker) 07-1988

Methodist Centre for Transplantation & Immunology of Reproduction Conference
"Can animal models help?" panel discussion - immunotherapy of recurrent abortion (Speaker) 08-1988

WHO Conference -
"Contraception and mechanisms of endometrial bleeding:macrophages and other migratory cells in endometrium; relevance to endometrial bleeding" (Speaker) 11-1988

3rd Banff Conference on the Materno-fetal Interface
"Role of factor related to transforming growth factor β2 in successful pregnancy" (Speaker) 01-1989

European Molecular Biology Organization
Basel Institute for Immunology
"SCID mice in reproductive biology" (Speaker) 02-1989

University of Ottawa Ob-Gyn, 1989 update in Reproductive and Infertility
"Immunology for Obstetricians & Gynecologists" (Speaker) 05-1989

St Mary's Hospital Medical School, London U.K. Aleck Bourne Lecture
"The Immunology of Pregnancy Failure" (Speaker) 05-1989

Society of Obstetricians and Gynaecologists of Canada, Quebec City
"Key role of local intrauterine suppression in preventing rejection of the conceptus" (Speaker) 06-1989

Wichita Conference - Cellular signals controlling uterine function, University of Kansas,
"The origin and role of cytokines determining success and failure in the

post-implantation period" (Speaker)	09-1989
Erice Conference, Sicily - 1st International Symposium on "Immunobiology of Normal and Diabetic Pregnancy" (Speaker)	03-1990
Walter Heap Centennial Symposium on Embryo Transfer, Guelph, "Cytokines in post-implantation success of the embryo" (Speaker)	04-1990
American Society for the Immunology of Reproduction, Chicago, "Success and Failure of the Fetal Allograft" (Speaker)	06-1990
VII World Congress on Human Reproduction Helsinki, Finland, "Cytokines determining the success or failure of pregnancy" (Speaker)	07-1990
Institute of Molecular Genetics, Dept. of Immunology, Prague, "Role of a TGF-β2-like factor in preventing rejection by LAK cells" (Speaker)	07-1990
Institute of Physiology, Prague, "Role of polyamines in the success or failure of *in vitro* fertilized embryos" (Speaker)	07-1990
Serono Symposia USA, Ville d'Esterel, Quebec, "Controversies in Reproductive Endocrinology: An Update for the Clinician" (Speaker)	10-1990
American Academy of Dermatology, Atlanta, Georgia, "Organization of the Immune System" (Speaker)	12-1990
INSERM Colloque, Paris, "TGF-β in human and mouse reproduction" and "Is there an early embryo suppressor factor?" (Speaker) (declined; post-doc. sent to give talks)	12-1990
St. Justine Center for Clinical Investigation and Research, Montreal, Quebec	

"Immunotherapy for Recurrent Miscarriage"
(Speaker) 04-1991

Society for Obstetricians & Gynecologists of
Canada, Toronto
"Immunology and Reproduction: Basic and
Applied" (Speaker) 06-1991

American Society for Reproductive Immunology
Symposium, Virginia, USA
"Immunotherapy for Unexplained Recurrent
Abortion: Problems in Proving Efficacy/Inefficacy"
(Speaker and Symposium Chairperson) 06-1991

Chicago Medical School,
University of Health Sciences, Chicago, Ill. USA
"Mechanisms of Suppression of Abortion (Rejection
of the Fetoplacental Unit): of Mice and Women".
(Speaker) 11-1991

University of Western Ontario
"Role of transforming growth factor β on the
regulation of trophoblast cell invasiveness, growth
and differentiation". (Speaker) 04-1992

National Institutes of Health,
Contraceptive Development Branch, NICHD,
Bethesda, Md., USA
"Cytokines: role in regulation of endometrial bleeding.
(Speaker) 05-1992

Colleges Biosciences Association
George Brown College,
Toronto, Ontario
"Immunology made simple - 1992 edition".
(Speaker) 06-1992

Canadian Society of Obstetricians &
Gynecologists, 48th Annual Meeting,
Vancouver, B.C.
"Anti-sperm antibodies: do they or don't they?"
"Objectives: Immunology for the Obstetrician
Gynecologist". (Speaker) 06-1992

University of Toronto
"TGF-β in pregnancy and cancer" (Speaker) 06-1992

8th International Congress of Immunology, Budapest, Hungary "Role of a unique species of TGF-β in preventing rejection of the conceptus during pregnancy". (Speaker)	08-1992
T Cell Sciences (Boston) "Novel form(s) of TGF-β in pregnancy and cancer" (Speaker)	09-1992
National Research Council, Biotechnology Institute, Montreal "Murine pregnancy decidua produces a unique immunosuppressive molecule related to TGF-β2" (Speaker)	11-1992
Endocrinology of Embryo-endometrial Interactions, Bordeaux, France Satellite Symposium of the 9th International Congress of Endocrinology (N.I.C.E.92) Bordeaux, France "Cytokines: molecular language for "yes" and "no". Invited speaker (declined due to lack of travel funds).	09-1992
Ontario Thoracic Society "Should you treat non-operable, non-small cell lung cancer?" (Speaker)	02-1993
AAI FASEB Meeting (Denver) "Murine and human decidua-associated natural suppressor cells release a novel type of TGF-β2". (Minisymposium Speaker)	05-1993
SCID Mouse Workshop University of Guelph, Guelph, Ont. "Abortion in SCID mice" (Speaker)	06-1993
Meta-analysis of immunotherapy of recurrent miscarriage (Washington, DC) (Speaker & Chairperson)	07-1993
Serono Symposia, Boston, MA "Novel transforming growth factor β's in pregnancy and cancer" (Speaker)	08-1993

RT Weaver Day, Hamilton, Ont
"Identification of suppressor cells in decidua that produce novel species of TGF-β suppressor factor in successful human pregnancies" (Speaker) 11-1993

British Society for Immunology "Mechanism of pregnancy - induced remission of arthritis" (Speaker) 12-1993

University of Newcastle upon Tyne
"TGF-β's in pregnancy and cancer". (Speaker) 01-1994

University of Cambridge, UK
"Novel TGFβ's in the uterus". (Speaker) 01-1994

University of Oxford, UK
"TGF-β's in pregnancy and cancer". (Speaker) 01-1994

Guy's Hospital Medical School, London, UK
"Pregnancy and Arthritis". (Speaker) 01-1994

Mohawk College Health Sciences Education
Current Topics of Interest in Immunology
"Review of the Immune System". (Speaker) 04-1994

INSERM-Merieux Colloque, France
"Effect of immune status on stress-induced abortion". (Speaker) 05-1994

Sunnybrook Hospital Oncology Dept.
"Is dose intensity dose insanity?
The problem of Stage IV breast cancer". (Speaker) 02-1995

MFIG-BSI Symposium,
"Psycho-neuroimmunological mechanisms in spontaneous abortion: of mice, women & James Mowbray
St. Mary's Hospital, London, UK. (Speaker) 04-1995

6th International Society for Immunology of Reproduction, Washington, D.C., USA
Local & systemic effects of pregnancy-related TGFβ
(Symposium Speaker) 07-1995

9th International Congress of Immunology,
San Francisco, USA
"At the interface: uterine immunoregulation during
pregnancy" (Symposium Speaker) 07-1995

The 16th Annual ASRI Meeting "γδ T cells as
determinants of pregnancy success or failure:
return of the "fetal allograft?"
(Symposium Speaker) 06-1996

The 13th Rochester Trophoblast Conference
and, The Thomas G. Wegmann Memorial Symposium
on Reproductive Immunology, Banff, Alberta
"αβ and γδ T cells and reproductive success/failure"
(Symposium Speaker & Member of Organizing
Committee) 09-1996

Third International Meeting "Mechanisms in Local
Immunity" Third Meeting of Alps-Adria Society for
Immunology of Reproduction
"γδ T cells and outcome of early pregnancy"
(Chair and Symposium Speaker) 09-1996

AAAAI/AAI/CIS Joint Meeting, San Francisco
"Maternal Uterine T Cell Determination of Survival
of the Fetus *QUA* Parasite
(Invited Symposium Speaker & Chair) 02-1997

17th Annual Meeting of ASRI, Vancouver
"Paradigm Lost, Paradigm Gained"
(Invited Speaker) 06-1997

Czech Academy of Science
"Paradigm shifts in immunology of reproduction:
New insights on role of T cells and on mechanisms
causing pregnancy failure"
(Invited Speaker) 07-1997

AAI Annual Meeting, San Francisco
"Maternal uterine T cell determination of survival
Of the fetus *qua* parasite"
(Invited Speaker and Chair) 04-1998

18th Annual Meeting of American Society
Reproductive Immunology
"Why did your mother reject you"?

(Invited Speaker)	05-1998
Fourth Meeting of Alps Adria Society for Immunology of Reproduction, Opatija, Croatia "Immunopathology of early pregnancy" (Invited Speaker and Chair)	09-1998
7th ICRI, New Delhi, India Tom Wegmann Memorial Horation "From trophoblast antigens and cells to cytokines: visions and realities" (Invited Plenary Speaker)	10-1998
VII International Congress of Reproductive Immunology "Immunological Contraception" Dr. B.R. Ambedkar Centre, New Delhi, India (Symposium Speaker)	10-1998
10th ICI New Delhi, India Th1/Th2,3 NK Cells: Regulation of FGL2 prothrombinase-mediated abortions? (Invited Chair & Speaker)	11-1998
10th International Congress of Immunology New Delhi, India "Immune regulation of maternal fetal interactions" (Invited Speaker and Chair - "Reproductive Immunology)	10-1998
American Society Reproductive Immunology 19th Annual Meeting "Molecular mechanism in immunologically-modifiable pregnancy failure" (Speaker)	06-1999
Frontiers in Reproduction Symposium, Wood's Hole 19th Annual Meeting NICHD/NIH (Invited Speaker)	07-1999
10th International Congress Mucosal Immunology Amsterdam, The Netherlands (Declined)	7-1999
European Placenta Meeting, Austria (Declined)	9-1999

European Congress in Immunology & Pregnancy
"Exploring the maternal fetal interface"
Rome, (Lead Speaker) 10-1999

MRC Group on Organ Injury, University Health Network,
The Toronto General Div.
"Transplantation tolerance mechanisms as a basis for successful pregnancy"
Toronto (Invited Speaker) 10-1999

Dartmouth Medical Centre,
"Maternal Recognition of the Fetal Allograft: Identification Of Immunogenetic Determinants determining rejection or Tolerance"
Lebanon, New Hampshire
(Invited Speaker) 03-2000

"Immunomodulation in Recurrent Abortion, Crohn's Disease, and Rheumatoid Arthritis
Clinical and Molecular Basis of Transfusion-Induced Immunomodulation
Washington D.C. (Invited Speaker) 03-2000

"The immunoregulatory tolerance-promoting molecule OX-2 expressed at the maternal-fetal interface prevents spontaneous abortions" (Invited Speaker and Chair)
6th Congress of the AASRI
Pecs, Hungary 06-2000

The Centre for Human Reproduction
Chicago, Illinois
"Behind the evidence of evidence based medicine as applied to obstetrics & gynecology. Would you believe this?" (Invited CME speaker) 11-2000

The Centre for Human Reproduction
New York City, New York
"TRIM: transfusion-induced immunomodulation as a treatment modality in infertility and recurrent miscarriage. What do we really know?"
(Invited CME speaker) 11-2000

University of Rome (Italy),
"New insights into molecular pathogenesis of human pregnancy success and failure derived from studies of mouse models".

(Invited speaker)	6-2001
VIII International Congress of Reproductive Immunology: Presidential Plenary Address "Thinking outside the box"	7-2001
Annual meeting of Japan Society for Immunology of Reproduction (Tokyo): (Invited International Lecturer) "New Insights into the Molecular Basis for Success or Failure of Early Pregnancy"	12-2001
Dept. Immunology/Microbiol. Chicago Medical School "New Insights into the Molecular Basis for Success or Failure of Early Pregnancy"	2-2002
Annual meeting of Czech Society for Immunology of Reproduction. "Danger and the fetomaternal interface" (Invited speaker: declined)	5-2002
Annual Meeting of the American Society for Reproductive Immunology (Chicago). "Molecular basis for 'danger' at the feto-maternal interface and its immunotherapeutic antithesis."	6-2002
8th Congress of AASIR (Weimar, Germany) "Not Th1 nor Th2 but 'danger' for whom the belle tolls"	9-2002
Brown University-Lingpong Meeting (Providence, RI) "Danger at the fetomaternal interface: the molecules and messengers."	11-2002

ADMINISTRATIVE RESPONSIBILITIES:

Department:	Promotion & Tenure Committee (member) MVIP Executive & Education Committees (member) Palliative Care Committee (Chairman) Clinical Program Management (member) Quality Assurance Sub-Committee (member) Library Users Committee (member)
University:	*Ad Hoc* CAWAR Committee (member) CAUWAR Reevaluation Committee *Ad Hoc* Pension and MUFF Committees Pension Trust Committee

	Pension Surplus Distribution Committee
Faculty:	Clinical Faculty Association (President) Faculty Association (CFA Representative) Regional Medical Associates (Member of Board)
HHSC:	Research Ethics Board (Member) Pharmacy and Therapeutics Committee (member)

MEMBERSHIPS IN PROFESSIONAL SOCIETIES:

Royal College of Physicians & Surgeons of Canada (Fellow)		1974-
Canadian Society for Immunology		1977-
Canadian Oncology Society		1978-
American Association of Immunologists		1978-
Royal Society of Medicine - London		1979-
Fellow		1992-
American Society for Reproductive Immunology		1983-
International Society for Immunology of Reproduction		1984-
Canadian Society for Clinical Investigation		1985-
Canadian Fertility & Andrology Society		1986-
British Society for Immunology		1988-
Canadian Association of Medical Oncologists		1991-
Society for the Study of Reproduction		1992-
American Society for Reproductive Medicine (Fellow)		1999-
Royal College of Physicians (Edinburgh, UK)(Fellow)	elected installed	2001 2002

Declaration under U.S.C.§ Rule 132

IN THE MATTER OF
US Patent Application No. 09/380,327
by ROBERTSON et al.

EXHIBIT DAC-2

This is Exhibit DAC-2 referred to in the Statutory Declaration by David Alexander CLARK

dated 16 Dec 2002

Before me:

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada.

PERGAMON

The International Journal of Biochemistry & Cell Biology 30 (1998) 293–298

The International Journal of Biochemistry & Cell Biology

Molecules in focus

Transforming growth factor-beta (TGF-β)

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[b]Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, University College London Medical School, 5 University St., London WC1E 6JJ, UK

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Abstract

The TGF-β family of cytokines are ubiquitous, multifunctional and essential to survival. They play important roles in growth and development, inflammation and repair and host immunity. The mammalian TGF-β isoforms (TGF-β_1, β_2 and β_3) are secreted as latent precursors and have multiple cell surface receptors of which at least two mediate signal transduction. Autocrine and paracrine effects of TGF-β's can be modified by extracellular matrix, neighbouring cells and other cytokines. The vital role of the TGF-β family is illustrated by the fact that approximately 50% of TGF-β_1 gene knockout mice die in utero and the remainder succumb to uncontrolled inflammation after birth. TGF-β_2 and TGF-β_3 gene knockout mice are not yet described. More recently, novel TGF-β-like molecules have been described which share some of the properties of the mammalian TGF-β isoforms. The role of TGF-β in homeostatic and pathogenic processes suggests numerous applications in the diagnosis and treatment of various diseases characterised by inflammation and fibrosis.

1. Introduction

Supernatants conditioned by tumor cells may enable phenotypically normal rat kidney fibroblasts (NRK cells) to grow as colonies in semisolid agar, a feature of malignant cells. Two cytokines mediate this effect, transforming growth factor (TGF)-α (5 kDa) and TGF-β (25 kDa) [11]. The 'gold standard' defining TGF-β is the ability to promote colony formation by NRK cells in semisolid medium in the presence of TGF-α or its equivalent, epidermal growth factor. However, the commonest bioassay for TGF-β is the inhibition of mink lung epithelial cell growth.

2. Structure

At least 23 distinct genes code for the TGF-β superfamily [1] and there exist at least five TGF-β isoforms. The amino acid sequence of TGF-β's is highly conserved between species. This article

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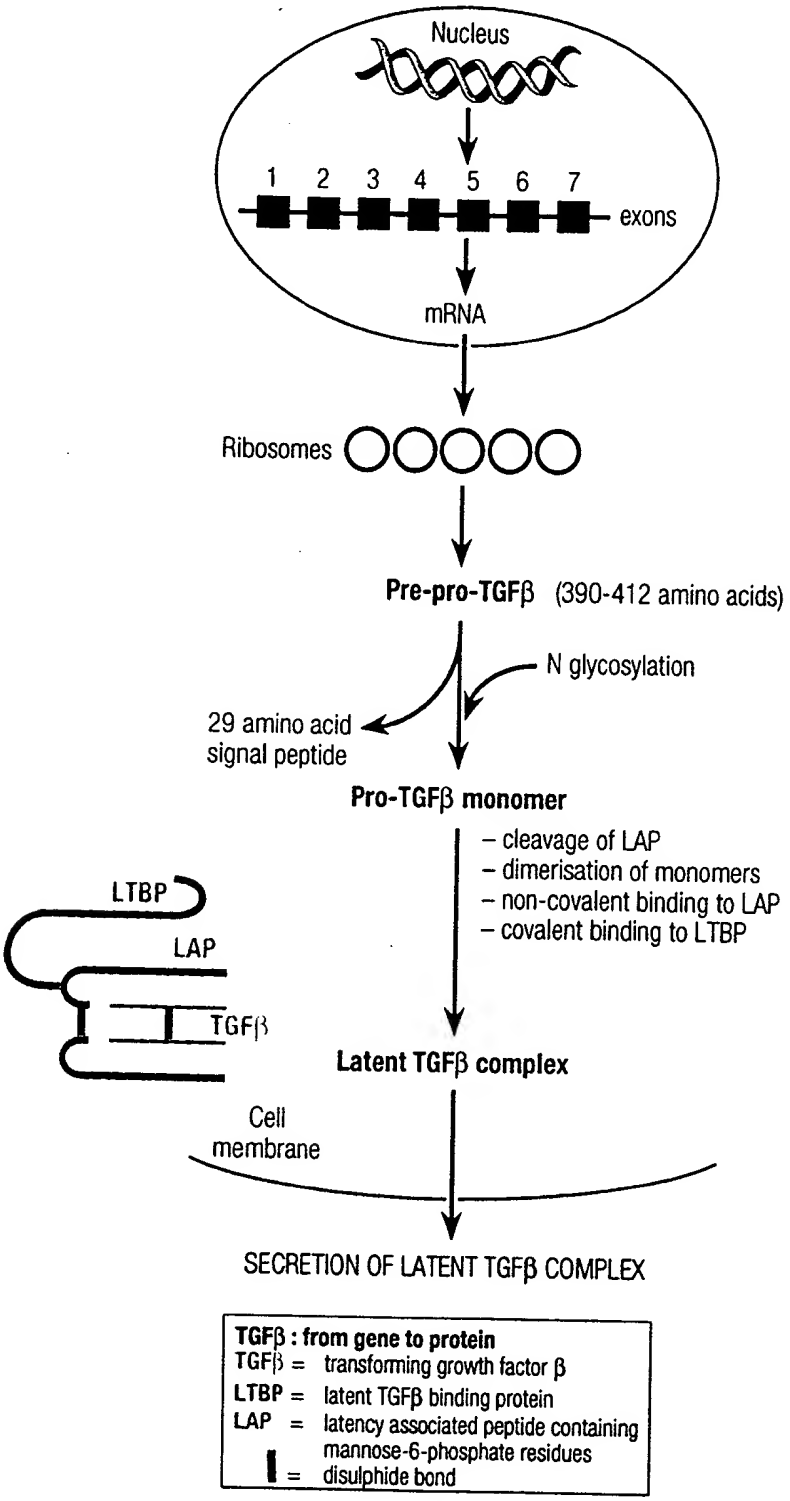

Fig. 1. Synthesis and secretion of TGF-β: From gene to released product. Details of intracellular processing is shown for TGF-β_1 for which most information is available. Other TGF-β isoforms are believed to follow a similar sequence. *aa denotes amino acid.

focuses on mammals where three TGF-β isoforms are described. The bioactive molecule is a homodimer of two 12.5 kDa peptides joined by a sulphidryl bond to form a 25 kDa molecule (TGF-$\beta_{1.1}$, -$\beta_{2.2}$, or -$\beta_{3.3}$). Heterodimers (e.g. TGF-$\beta_{1.2}$ and -$\beta_{2.3}$) may also form. Some bioactive TGF-β's have atypical molecular weights. These include 120/90 kDa glioblastoma-derived factors, 40 and 30 kDa molecules in human breast cancer cell homogenates and 30–32 kDa and 15–20 kDa factors released by uterine lining cells of pregnant mice [2]. These atypical molecules differ functionally from the TGF-β_{1-3} isoforms and may therefore offer valuable insight into the structure–function relationships of the TGF-β isoforms.

3. Synthesis and degradation

The synthesis of latent TGF-β is illustrated in Fig. 1. The details of intracellular processing are most completely defined for TGF-β_1. The TGF-β_1 and -β_3 genes span 100 kb and -β_2, 70 kb. The cleavage of pro-TGF-β is mediated by endopeptidases such as furin [4] to yield the 12.5 kDa monomer. The cleaved fragment dimerizes to form a 75 kDa latency binding peptide which assists in folding of the 25 kDa dimer and remains associated to form the latent complex. A 135 kDa latency binding peptide bearing mannose-rich carbohydrate may attach to the complexed latency peptide dimer. The latent complex may be stored in α granules in platelets (the largest reservoir of TGF-β in the body), or may be secreted. Secretion is enhanced by the carbohydrate. It is unclear if TGFβ's of atypical molecular weight arise from different genes than do the 25 kDa TGF-β_{1-3} or from a different processing of the same pro-peptide.

On release, latent TGF-β may bind to a cell membrane-associated mannose-6-phosphate receptor or to extracellular matrix, as illustrated in Fig. 2. Release of bioactive TGF-β by neoplastic and non-neoplastic cells (such as macrophages and immunosuppressor cells) may occur by activation of latent TGF-β by simultaneously released serine protease(s) [5]. Similar enzymatic processes may explain activation of latent TGF-β where one type of cell produces latent TGF-β (e.g. vascular endothelial cells) and a second cell type (e.g. pericytes) causes activation. Several different enzymes may activate latent TGF-β bound to extracellular matrix proteins.

Bioactive TGF-β's may bind to matrix, to α_2-macroglobulin and to decorin (all of which usually inhibit activity), or to carriers such as albumin and newly synthesized IgG (which does not inhibit activity). α_2-macroglobulin may facilitate TGF-β action on cells bearing α_2-macroglobulin receptors. TGF-β–α_2-macroglobulin complexes are taken up via hepatic mannose-6 phosphate/insulin-like growth factor II receptors and possibly catabolized. Bioactive TGF-β's may also be degraded by proteases and elastases released at sites of inflammation [2]. TGF-β may be excreted in the urine.

4. Biological functions

The TGF-β's play roles in adult and embryonic growth and development, in inflammation and repair including angiogenesis and in regulation of host resistance mechanisms. TGF-β's have both autocrine and paracrine effects. Biological effects are mediated via receptors. Six different receptors have now been described, the most recent being endoglin [7, 13]. The receptors may be subdivided into those which mediate an intracellular signal and those which store and/or present TGF-β's to signaling receptors. Type II receptors, to which TGF-β is bound, associate with type I receptors to form a signaling complex. The signal appears to proceed via a series of MAD peptides [3] and can result in inhibition or stimulation. For example, adaptive responses (cytotoxic T-cells, delayed-type hypersensitivity/cellular immunity T-cells and helper T-cells) may be inhibited as may be the activity of natural effector cells such as macrophages, natural killer cells and natural cytotoxic cells which contribute to host resistance. Epithelial and endothelial cell growth *in vitro* is also inhibited. In general, mesenchymal cells are stimulated by TGF-β's but the receptor(s) which mediate proliferation and

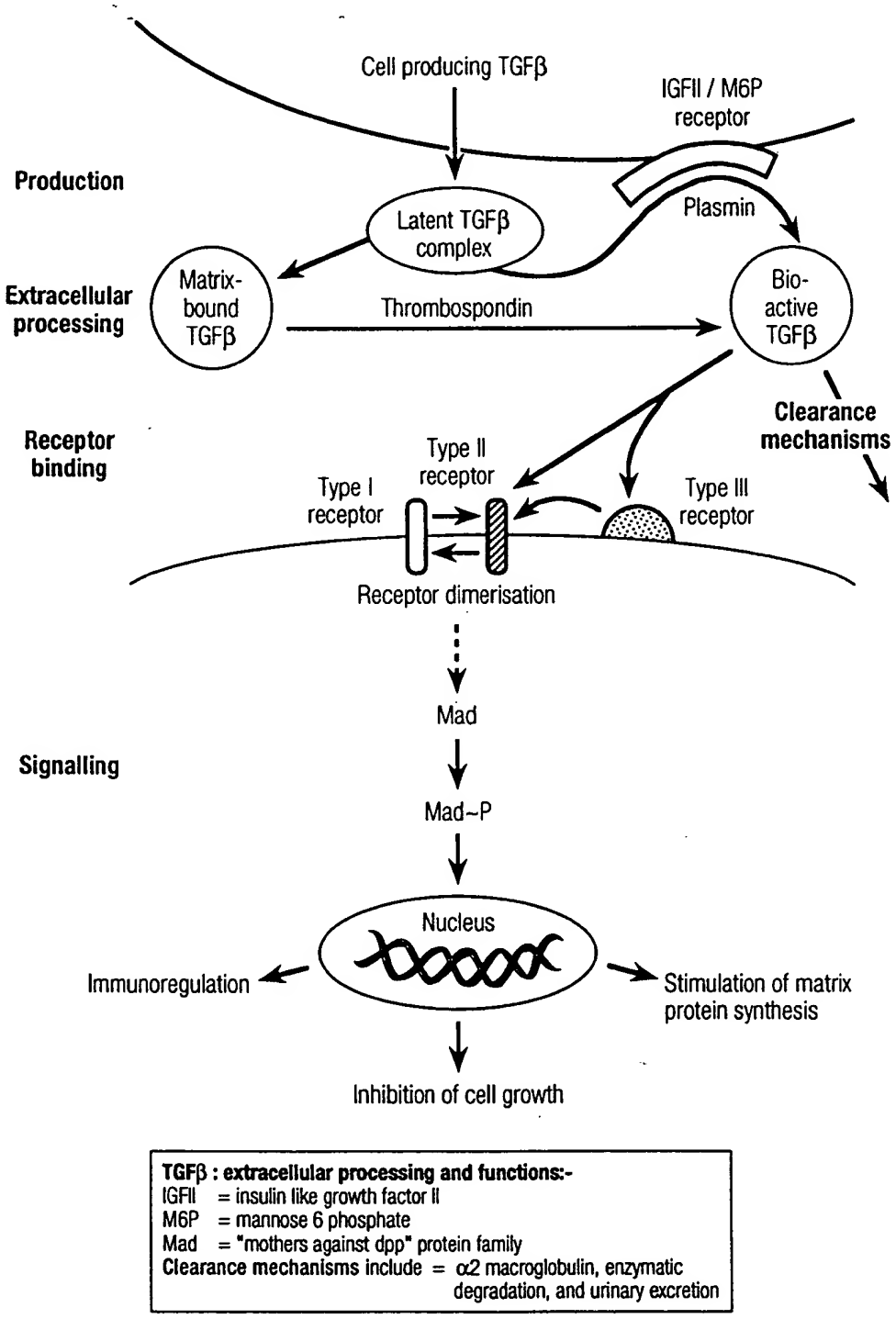

Fig. 2. Extracellular pathways of TGF-β: From release of latent form to action on receptors and catabolism. For details, see text.

synthesis of matrix proteins such as procollagen, together with the promotion of monocyte and neutrophil influx into inflammatory sites, are not yet clearly defined. Epithelial tumor cells lacking type I and II receptors are unusual in that they proliferate in response to TGF-β's. It is possible

that type V receptors (which have kinase activity as do type II receptors) mediate some of these effects. Neutrophil influx into tissues may be inhibited by TGF-β, rather than stimulated, as in TGF-β-mediated prevention of reperfusion injury. The response depends on TGF-β concentration and TGF-β-mediated alteration in fibronectin production [2, 10]. Similarly, dose-dependent reversal of TGF-β effects on endothelial cell growth have been attributed to variations in surface receptor phenotype and modulation of acidic fibroblast growth factor receptors [8].

Whilst the pro-inflammatory and fibrogenic effects of TGF-β have been called the 'dark side' of its character, TGF-β enhances wound strength and stimulates epithelial cell growth *in vivo*. This contrasts with the inhibitory effect seen *in vitro* and promotes healing. Epithelial cells in this regenerative setting resemble malignant tumor cells which usually proliferate in response to TGF-β. TGF-β can stimulate as well as inhibit immune responses [2, 6, 12]. TGF-β_1 gene knock-out mice establish the importance of TGF-β's in normal homeostasis. TGF-β_1 gene knock-out embryos have a 50% intrauterine death rate and the homozygous progeny develop a fatal inflammatory syndrome by 3 to 6 weeks of age. No studies of TGF-β_2 or TGF-β_3 knock-out mice have yet been reported.

Several factors appear to influence the response to bioactive TGF-βs. The potency of TGF-β_1, -β_2 and -β_3 is similar in stimulating NRK cell colony formation but TGF-β_2 is much less inhibitory to bone marrow cell growth compared with TGF-β_1 on a per molecule basis and TGF-β_3 is three to five times more potent in stimulating osteoblast growth and collagen synthesis. Particular TGF-β isoforms are required for certain embryological events (e.g. TGF-β_3 for murine palate development). The structure of the TGF-β molecule and the nature of TGF-β binding proteins and receptors which lack kinases needed for direct cell signaling are important. Endoglin mediates the growth inhibitory response to TGF-β_1 and -β_3 on cells such as fetal trophoblasts and may inhibit trophoblast invasion of maternal uterine tissues [13]. TGF-β binding proteins such as endoglin present the TGF-β molecule to the receptors with signaling activity. Invasive cells such as fetal trophoblasts produce metaloproteinases which may liberate and activate inactive matrix-associated TGF-β. Other cytokines may also enhance or inhibit the effects of TGF-β [9]. TGF-β_3 may be less fibrogenic *in vivo* and antagonize the fibrogenic effects of TGF-β_1 and -β_2 [12]. As an illustration of the role of the cellular environment on response, replication of NRK fibroblasts or epithelial cells grown as anchorage-dependent monolayers in culture is inhibited by TGF-β, but is stimulated when the same cells are grown in an anchorage-independent manner. *In vivo*, TGF-β enhances wound reepithelialization. These observations have led to the concept that cytokines and matrix proteins act together as a molecular language modulating target cell responses. Defining these interactions should facilitate our understanding of seemingly paradoxical effects. It is also clear that one cytokine may modify receptors for the same type of cytokine and receptors for other cytokines on target cells.

5. Possible medical applications

Applications of TGF-β's remain speculative. Enhancing wound healing and healing of lesions in inflammatory bowel disease appears promising. Recombinant simian TGF-β_2 has been used systemically as an immunosuppressant (e.g. for multiple sclerosis where enhanced TGF-β levels herald remission) but may entail renal toxicity. The commercial use of TGF-β_{1-3} is therefore still awaited. TGF-β's may prevent post-ischemia reperfusion injury and could be valuable following myocardial injury and cerebrovascular ischaemia. Some women appear to 'reject' their embryos due to subnormal production of atypical TGF-β-like molecules, suggesting diagnostic and therapeutic approaches. Inhibition of TGF-β could be useful in arresting development of pathologic states associated with fibrosis such as liver cirrhosis, pulmonary fibrosis, scleroderma, chronic pancreatitis and nephritis. TGF-β levels may be elevated by hemorrhage or infection, and could provide useful therapeutic targets. In sum-

mary, the TGF-β family constitute a fascinating set of molecules greatly expanded by the recent discovery of TGF-β's with atypical molecular weights and distinct functional properties the potential of which remains to be tapped.

References

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[2] D.A. Clark, K.C. Flanders, H. Hirte, J.R. Dasch, R. McAnulty, R.J. McAnulty, G.J. Laurent, Characterization of murine pregnancy decidua transforming growth factor β. I. Transforming growth factor β_2-like molecules of unusual molecular size released in bioactive form, Biol. Reprod. 52 (1995) 1380–1388.

[3] R. Derynck, Y. Zhang, Intracellular signalling. The Mad way to do it, Current Biol. 63 (1996) 1226–1229.

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Declaration under U.S.C.§ Rule 132

IN THE MATTER OF
US Patent Application No. 09/380,327
by ROBERTSON et al.

EXHIBIT DAC-3

This is Exhibit DAC-3 referred to in the Statutory Declaration by David Alexander CLARK

dated 16 Dec 2002

Before me:

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada.

16/12/92 David A Clark DR23

BIOLOGY OF REPRODUCTION 52, 1380–1388 (1995)

Characterization of Murine Pregnancy Decidua Transforming Growth Factor β. I. Transforming Growth Factor β2-Like Molecules of Unusual Molecular Size Released In Bioactive Form[1]

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ABSTRACT

A novel type of bioactive transforming growth factor β2 (TGFβ2)-related immunosuppressive activity, lower in molecular mass (20–23 kDa) than a conventional TGFβ2 standard (25 kDa), has been shown to be released by non-T non-B suppressor cells of murine decidua into fetal bovine serum (FBS)-containing tissue culture medium during a 48-h incubation at 37°C. Substitution of a serum-free medium has allowed direct PAGE-Western blotting and the demonstration in supernatant (prior to incubation at 37°C) of a TGFβ2-immunoreactive doublet at a higher molecular mass (26 and 27 kDa) than the standard but with apparent immunosuppressive activity. When decidua were incubated in serum-free medium at 37°C, immunosuppressive activity in the supernatant increased to peak at 18 h in association with the appearance of the previously described lower molecular mass species of molecule. The doublet did not appear to be the result of glycosylation of a conventional 25-kDa TGFβ2 and could be converted into the lower molecular mass form by incubation for 18 h at 37°C, with pH 4.5 but not with pH 7.5 buffer; this incubation in the absence of decidual cells was not accompanied by increased immunosuppressive activity. This transformation could be blocked by a brief heating of the supernatant to 80°C for 10 min to destroy enzymes present in supernatant prior to mixing with the acidic buffer, but biologic activity neutralizable by anti-TGFβ2 was retained. These data suggest that TGFβ2-like immunoregulatory activity in murine decidua is biologically active and that the size of the molecules may be modified by local enzymatic activity. The immunosuppressive factor was shown to be capable of stimulating procollagen production by confluent human fibroblasts assayed by HPLC measurement of hydroxyproline; but, in comparison to an authentic TGFβ2 standard, the potency was much lower. On this basis, it would be possible to have local active immunosuppression without stimulation of fibrosis in implantation site decidua.

INTRODUCTION

We recently described a transforming growth factor β2 (TGFβ2)-related molecule produced by small-sized non-T suppressor cells at the implantation site in murine decidua [1, 2]. A major peak of suppressive activity, purified by HPLC using a molecular sieving column and high potassium-acetic acid (pH 2.9) buffer, proved by Western blotting and eletroelution to have a molecular mass slightly lower than that of a purified TGFβ standard [1, 2]. Nevertheless, these molecules were able to stimulate anchorage-independent growth of normal rat kidney (NRK) fibroblasts in semisolid medium in the presence of epidermal growth factor (EGF); the bioactivity of these molecules was neutralized by anti-TGFβ2; and release of the activity correlated closely with expression of TGFβ2 mRNA by small lymphocytic suppressor cells in decidua [1, 3]. Two problems with these data have been identified. First, the supernatants were generated by incubation of disaggregated decidua at 37°C for 48 h in tissue culture medium containing 10% non-heat-inactivated fetal bovine serum (FBS): latent TGFβ might have been activated by enzymes during the incubation period, and/or conventional TGFβ might have been modified so as to have a lower molecular mass. Indeed, Wollenberg found that incubating radioiodinated TGFβ1 with proteases and elastases generated fragments in the size range of our activity as defined by PAGE-Western blots and electroelution [4]. A second problem related to the use of medium containing 10% FBS was the high protein concentration, which made it difficult to determine the size of the molecules by Western blotting without a preliminary HPLC purification: the acid buffer conditions could have modified the TGFβ species present in decidual supernatants.

In this paper, we show that our TGFβ2-related activity is present in serum-free medium incubated with decidua. Active TGFβ2 was present in the medium in which the cells were suspended, without incubation at 37°C (time = 0), as a doublet with molecular masses slightly greater than that of TGFβ2. This doublet did not appear to reflect glycosylation of a conventional 25-kDa TGFβ2. With incubation of the decidual tissue, activity increased and peaked at 18 h, and this was associated with increased antigenic and biologic activity at the lower molecular mass (20–23 kDa) and an alteration in the doublet. Bioactivity included immu-

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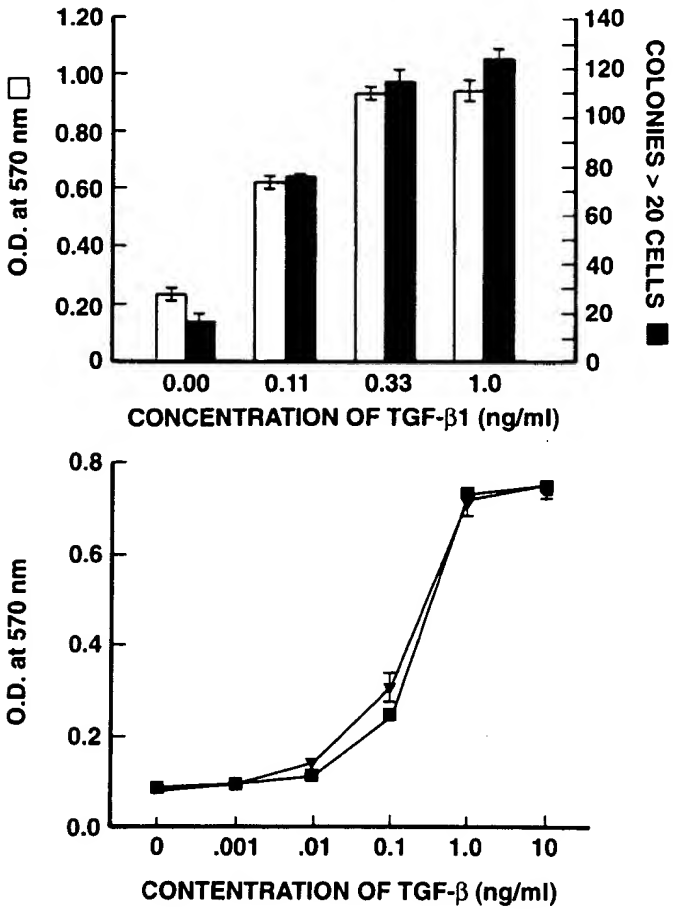

FIG. 1. NRK fibroblast assay for TGFβ. Upper panel: Relationship of colorimetric end point to colony count performed with a microscope. No significant difference was detected by Student's *t*-test for paired samples ($p > 0.5$, 4 replicates per group). Error bars represent ± 1 SEM. Addition of rabbit anti-TGFβ antibody, 10 μg/ml, completely abrogated growth-stimulating effect of TGFβ at all test concentrations (data not shown). Lower panel: comparison dose response of TGFβ1 (solid squares) to TGFβ2 (solid inverted triangles). Error bars represent ± 1 SEM, 4 replicates per point.

have found this to directly correlate with colony formation and to provide a less tedious assay. Briefly, 25 μl tetrazolium (MTT; Sigma, 5 mg/ml in PBS) was added to each well, and samples were incubated at 37°C for 4 h. Then 100 μl extraction buffer (20% [w/v] SDS in 50% dimethylformamide in distilled water containing 2% glacial acetic acid and 2.5% 1 N HCl, pH 4.7) was added to each well to solubilize formazan generated by the viable cells. After overnight incubation at 37°C, the OD of each well at 570 nm was determined by means of an EMAX ELISA reader (Molecular Devices, Palo Alto, CA) using a reference OD of 630 nm. The difference in OD was proportional to the number of colonies present as shown in Figure 1 (upper panel). Purified TGFβ1 obtained from R & D Systems (Minneapolis, MN) was routinely used to generate a standard curve, as the result was not different from that obtained with porcine TGFβ2 (as shown in Fig. 1, lower panel) and the latter was in short supply.

Affinity column- and HPLC-purified immunosuppressive activity was also tested for its ability to stimulate procollagen production by fibroblasts through use of a method described previously [10]. Briefly, human fetal lung fibroblasts (HFL-1, obtained from ATCC) were cultured to confluence in 12-well plates with DMEM-5% newborn calf serum (NCS). They were then preincubated for 24 h with medium containing 4 mM glutamine, 50 μg/ml ascorbic acid, 0.2 mM proline, and 2% NCS. The medium was replaced with medium containing the HPLC fraction or TGFβ2 (either natural porcine or recombinant simian). The cells were incubated for a further 24 h before harvesting. After harvesting, proteins in the cell layer and medium were precipitated with 67% ethanol and the samples filtered (0.65 μm). The ethanol-insoluble fraction was then hydrolysed in 6 M hydrochloric acid, and the hydroxyproline present was measured by HPLC as an index of procollagen content. A dose-response curve was generated for natural porcine TGFβ2. These results were then compared with the response to human recombinant TGFβ2 (in PBS or HPLC buffer) and to the purified decidual factor at dilutions of 1/10 and 1/20. The effect of HPLC buffer at 1/10 and 1/20 was also tested.

Modification of Supernatants

Serum-free conditioned supernatants were treated with *N*-glycanase (Sigma and Genzyme, Boston, MA) or endoglycosidase H (Genzyme) by the method of Sha et al. [11] prior to Western blotting to determine whether the position of the TGFβ2-like bands could be shifted. Briefly, 250 μl supernatant was added to 100 μl 10 mM EDTA, 0.2% SDS, 0.1 M 2-mercaptoethanol, and 1% NP-40 buffer containing 1.25 mU of *N*-glycanase, and incubation was conducted for 18 h at 37°C. For endoglycosidase F, a buffer containing 50 mM Na acetate, 2 mM CaCl, 2 mM EDTA, and Tris-HCl, pH 4.5, was used. The material was lyophilized and stored at 4°C until reconstitution in concentrated form in SDS buffer for PAGE-Western blotting.

In some experiments, the supernatants were heated at 80°C for 10 min (to activate any latent TGFβ and to inactivate endogenous enzymes [12]), and the effect of subsequent incubation at 37°C at acid pH was studied. Latent TGFβ was also activated in some experiments by addition of 5–10 μl of 1 N HCl to 100 μl supernatant (pH reached 2.0) at room temperature for 30 min [13] and then neutralizing to pH 7.2 by addition of 2 N NaOH.

Statistics

The significance of the difference between means was determined by Student's *t*-test.

RESULTS

Activity in Serum-Free Medium

Figure 2 illustrates the typical increase in suppressive activity in serum-free supernatants incubated for different times

nosuppression, stimulation of NRK fibroblast colony formation, and, to a lesser degree, stimulation of procollagen synthesis. The data are compatible with production and generation of novel bioactive TGFβ2-like molecules by murine pregnancy decidua.

MATERIALS AND METHODS

Mice and Supernatants

Female C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and mated with similarly obtained DBA/2 males as previously described. Day 0.5 was defined as the morning the vaginal sperm plug was sighted. On Days 12.4–14.5 of pregnancy, the animals were killed, the uteruses removed and opened, and the placentae peeled from the underlying decidua. The decidua was then harvested by scraping down to muscle and was disaggregated by passage through a 60-mesh stainless steel screen [1]. Details concerning cell yields and viability have been reported previously [1]. All procedures were carried out through use of α-MEM (Grand Island Biological Company, Burlington, ON, Canada) containing 10 mM HEPES and antibiotics but with 1 mg/ml (0.1%) BSA (Sigma, St. Louis, MO) in place of FBS. Usually one uterine decidua per milliliter was cultured in a Falcon #2057 (Los Angeles, CA) tube for varying time periods. Zero time (t0) supernatant (no 37°C incubation) was obtained by spinning down the cells in the 1-ml resuspension volume before incubation; t6, t12, t18, t24, t36, and t48 supernatants were similarly obtained by harvesting the 1-ml cell-free supernatant after the indicated time at 37°C in 5% CO_2. The t0 sample was at room temperature for approximately 30 min and would be expected to contain free TGFβ as well as molecules released from matrix and from cells disrupted during the disaggregation procedure.

Fractionation of Supernatants and Western Blotting

Serum-free conditioned medium was concentrated by speed vacuum centrifugation, and a 5–10-times concentrated sample was separated by HPLC using a TSKG2000 analytical column run at 1 ml per min as previously described [1]. Elution buffer was 0.05 M KCl, 0.58% acetic acid (pH 2.9); the reduction in concentration of KCl from 0.1 M made it easier to analyze speed vacuum-concentrated HPLC fractions by PAGE-Western blots. Western blotting of unseparated supernatant and HPLC fractions was done as described previously using a rabbit antibody specific for a peptide corresponding to amino acids 50–75 of TGFβ2 [1, 3, 5]. Prestained high molecular mass standards were obtained from Bethesda Research Labs (Bethesda, MD), and purified porcine TGFβ2 was obtained from R & D Systems (Minneapolis, MN). In some experiments, supernatant was applied to a 5-ml column containing Reactibeads (Pierce Chemical Company, Rockford, IL) to which the 3C7 murine monoclonal anti-TGFβ2 antibody had been attached [6]. The 3C7 antibody is known to neutralize murine decidua-associated suppressive activity by at least 75% [1]. Briefly, approximately 45 mg of purified antibody was dialyzed overnight against 0.05 M borate, 0.1 M NaCl buffer, pH 8.5. Reactigel 6X was washed with ice-cold deionized H_2O; 15 ml of packed gel was mixed with the antibody. After 29 h at room temperature, the gel was centrifuged and the supernatant removed. Twenty milliliters of 0.15 M glycine in 0.1 M bicarbonate buffer, pH 8.3, was added for 4 h to quench any residual active sites on the gel. The gel was washed with 3 alternating cycles of 0.1 M acetate + 0.5 M NaCl buffer (pH 4.3) and 0.1 M bicarbonate + 0.5 NaCl buffer (pH 8.3). Coupling efficiency was estimated to be approximately 95%. The 3C7-coated beads were loaded into a column, and decidual supernatant was added and incubated for 30 min at 22°C. Then the affinity column was washed with PBS until no optical density (OD) at 280 nm (OD 280) activity could be detected and was eluted with 0.1 M glycine, pH 2.9. One-milliliter fractions were collected and tested for OD 280 activity, antigenic activity as revealed by Western blotting, and biological activity as described below.

Bioassays

Immunosuppression was assessed through use of the murine CTL generation assay system previously described [1, 3]. Percentage ^{51}Cr-release values (P) were converted into the Nαt parameter linearly related to CTL number from the formula,

$$N\alpha t = \ln (1 - P/100).$$

Percentage suppression was calculated from 100−100 (Nαt with supernatant/Nαt in medium or buffer control) [3]. Recombinant human TGFβ2 standard and 3C7 murine monoclonal neutralizing antibody were kindly supplied by Celtrix Pharmaceuticals (Santa Clara, CA).

In some experiments, TGFβ activity was assayed by a modification of the NRK rat kidney fibroblast colony formation assay [1, 7–9]. The TGFβ assay employed the NRK-49F rat kidney fibroblasts (ATCC, Rockville, MD), which form colonies in semisolid medium containing EGF when TGFβ is added. NRK fibroblasts were maintained in monolayer culture in DMEM/F12 medium (Gibco, Grand Island, NY) containing 3% FBS (Gibco), 5 μg/ml insulin, 10 μg/ml transferrin, 5 ng/ml EGF, 5×10^{-5} M phosphoethanolamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (all additives from Sigma). The fibroblasts were harvested by trypsinization, and 500 cells in 50 μl preheated medium containing 0.3% low-melting-point agarose were plated in culture wells of a 96-well plate over a layer of 100 μl medium containing 1% low-melting-point agarose that had gelled at 4°C. After the NRK fibroblast aliquot had been similarly gelled, 50 μl of control or test sample was added and the plates were incubated at 37°C in a 5% CO_2 incubator for 7 days. Colony growth was determined by means of the colorimetric endpoint (through use of a modification of the method of Hansen et al. [8]), as we

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that detect the 6.0-, 5.0-, and/or the 3.5-kb-sized mRNAs hybridize with placental and/or uterine epithelial cells rather than with the decidual natural suppressor cells we have described [3, 29–34]. Further, different sizes of mRNA transcripts for vascular endothelial growth factor (VEGF) produce molecules of different molecular mass [35]. Cloning and expressing particular mRNA transcripts for decidual TGFβ2 could generate the types of TGFβ2-like molecules we have described in this paper. However, TGFβs are translated into a large precursor molecule within the cell, and this is modified by processing mechanisms prior to release from the cell surface [18]. It is therefore possible that the molecules of interest would be produced only if the particular mRNA were expressed in the 1E5.B5.1 subset of decidual lymphomyeloid cells. Since these have not yet been cloned, such an experiment would not likely be feasible (it has been suggested that uterine epithelium in pregnant mice may produce TGFβ2 [3, 33], but the types of molecules illustrated in Figure 3 can be obtained from the implantation site described, where there is no epithelium (Clark et al., unpublished results). The second approach is to purify each component to homogeneity for microsequencing and assay of biologic activities. Development of antibodies specific for the different sizes/types of the molecule will likely be required for a proper understanding of events in situ, and this may be possible once the molecular sequence is known [6, 20]. Enzymatic modification may play an important role in rendering the doublet more active. If enzymatic processing by decidua-associated enzymes does prove important in altering the biological activity of these TGFβ2-related molecules in situ, it will also be necessary to characterize the regulation of the enzyme in order to come to a full understanding of the biology of the TGFβ2-related activity. A variety of enzymes are known to be present at the fetomaternal interface [36].

Support for the existence of TGFβ species that do not have a molecular mass of 25 kDa, as does conventional TGFβ, is provided by studies of "successful neoplasms" other than the fetus. Glioblastoma cells have been reported to secrete an 80–100-kDa immunoprecipitable and bioactive TGFβ2, and breast cancer cells may contain a 30–32-kDa doublet as well as a 40-kDa TGFβ [37, 38]. Anomalous-sized bioactive TGFβs may not show the typical 12.5-kDa band on exposure to reducing agents [18]. Recombinant TGFβ2 of the conventional 25-kDa molecular mass injected into the peritoneal cavity of pregnant mice at 1–2 μg/day to produce immunosuppression generates thick adhesions and a sick animal (Clark et al., unpublished results). Fibrosis and inflammation (i.e., monocyte recruitment) seen with injection of the active recombinant TGFβ [39, 40] are not evident in histological sections of decidua where our putatively active TGFβ2-like factor is being produced [3]. Although the basis for this may lie in the binding of active TGFβ by matrix components (i.e., α-2 macroglobulin-pregnancy zone protein, PP14, α-fetoprotein, type IV collagen, and decorin) or by presence of other cytokines, which may modify TGFβ bioactivity [24, 25, 41–44], it is also possible that the TGFβ2 molecules produced in pregnancy decidua that have anomalous molecular mass are also biologically different from conventional TGFβ2. Figure 9 suggests that our molecules are less potent in stimulating collagen synthesis compared to immunosuppressive activity. It is known that TGFβ2 is less potent than TGFβ1 in inhibiting lymphomyeloid and endothelial cells [45–47], so variation in the potency of the many different effects of TGFβs [18] might be expected to arise with variation in molecular structure. Indeed, distinct functional domains have recently been identified on conventional 25-kDa TGFβ [48]. Whether our anomalous-sized TGFβ2-like molecules arise from a different gene than that coding for conventional 25-kDa TGFβ2, or arise by alternate splicing and posttranscriptional modification of mRNA from the known TGFβ2 gene, remains uncertain. Further studies of the novel decidual TGFβ2-related molecules will likely provide valuable insights into their role in successful reproduction.

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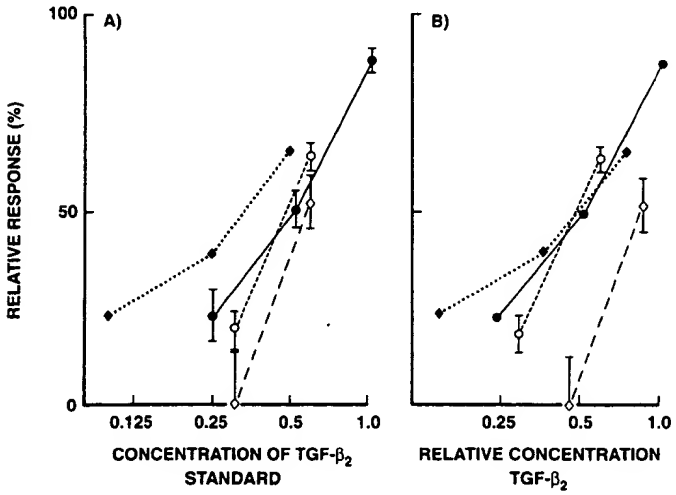

FIG. 9. Comparison of potency of TGFβ2 standards and purified decidual suppressor factor in the CTL suppression assay and the procollagen stimulation assay. **A)** Effects expressed as percentage of maximal for CTL suppression with TGFβ2 standard (solid circles) or HPLC fraction (open circles) with superimposition of the latter to allow readout of approximate effective concentration of TGFβ2. Solid diamonds show dose-response curve obtained with TGFβ2 standard in the procollagen stimulation assay and the effect of HPLC fraction (open diamonds) based on effective TGFβ2 concentration determined in the CTL suppression assay. Values represent mean ± 1 SEM. For procollagen stimulation assay, SEM values of points on standard curve were < 2%. **B)** Comparison of activity of HPLC-fractionated decidual factor in the CTL and procollagen stimulation assay after correction for differing sensitivities. The two standard curves (solid diamonds, solid circles) have been superimposed (i.e., the ED_{50}% values have been superimposed); consequently, the HPLC titration curve from the procollagen stimulation assay has moved to the right.

sitive than the CTL suppression assay, but the opposite was suggested by the purified decidual material. Correcting for differences in assay sensitivity by superimposing the two dose-response curves obtained with conventional TGFβ2 (Fig. 9B) created a more striking separation of potency of the decidual material in the two assays. The purified decidual material appeared to have only half of the expected potency in the procollagen synthesis stimulation assay. By simple inspection it is evident that one could have a significant level of immunosuppression at concentrations that did not significantly promote collagen synthesis.

DISCUSSION

The data in this paper show that the TGFβ2-like molecule released by murine pregnancy decidua has components that are higher as well as components that are lower in molecular mass than conventional 25-kDa bioactive TGFβs. The existence of a higher molecular mass doublet was overlooked in earlier studies because we used 48-h supernatants (generated in 10% FBS-supplemented medium). However, scrutiny of the published figure suggests that at least one component of the doublet was probably present as a faint band [1], consistent with Figure 3. The doublet appears to possess intrinsic biological activity in the absence of modification by proteases, low pH, or other factors involved in generating the lower molecular mass activity at 20–23 kDa. Further, affinity separation using solid-phase 3C7 anti-TGFβ2 suggested that part of the doublet did not adhere to the antibody and this component did possess suppressive activity. While part of the doublet present in t0 supernatant behaved similarly and did not bind to 3C7, most of the t0 activity was retained on the column and could not be eluted under the conditions used in these experiments; the latter phenomenon is currently under investigation. The ability of 3C7 to react with t0 activity was confirmed by neutralization of t0 activity in vitro. These data suggest that the molecules constituting the doublet in the t18 supernatant differed from those present at t0 due to alteration during the process of generation of the lower molecular mass species during the 18-h incubation. Alteration in TGFβ during processing may have precedent in that antibodies have been developed that distinguish between the intracellular and the extracellular form of TGFβ1 [20].

The origin of the activity in t0 supernatant (wash medium) is uncertain. It could represent molecules free in interstitial fluid or loosely bound to matrix, or it could represent intracellular TGFβ2-related peptides released during the disaggregation procedure (viability is approximately 60% [1]). Availability of antibodies specific for the different molecular mass species that could readily resolve this issue are not yet available. The increase in bioactivity during the first 18-h incubation at 37°C could represent release of additional activity due to cell death or synthesis and secretion of new molecules by viable cells. Sequential viability determinations during incubation would not likely distinguish between these alternatives because the cells responsible for TGFβ-mediated suppressive activity in vitro appear to be a minor subset of the lymphomyeloid cells present in the decidual cell preparation carrying the 1E5.B5.1 determinant of bone marrow-derived natural suppressor cells [3, 21, 22]. Preliminary pulse-chase studies using radiolabeled amino acids in vitro suggest that the doublet is actively synthesized [23], and this will be the subject of a future report. Cessation of net increase in active factor after 18–24-h incubation could be due to increased rates of degradation, cessation of synthesis, production of molecules such as decorin that may bind and antagonize the activity of TGFβs [24, 25], or a combination of some of these. Some decrease in synthesis would be expected due to cell death and due to the dependence of decidual suppressor cell function in vivo on soluble signals from fetal trophoblast cells that are absent in our in vitro culture system [26–28].

The demonstration that pregnancy-associated TGFβ2 may be composed of a spectrum of molecules of differing molecular mass not attributable to glycosylation suggests that achieving full understanding of their molecular structure and their individual properties will require one of two possible strategies. It is known that uterine TGFβ2 mRNA on Northern blots may have molecular sizes of 6.0, 5.0, 4.2, 3.5, and 1.5 kb; and there is some evidence to suggest that probes

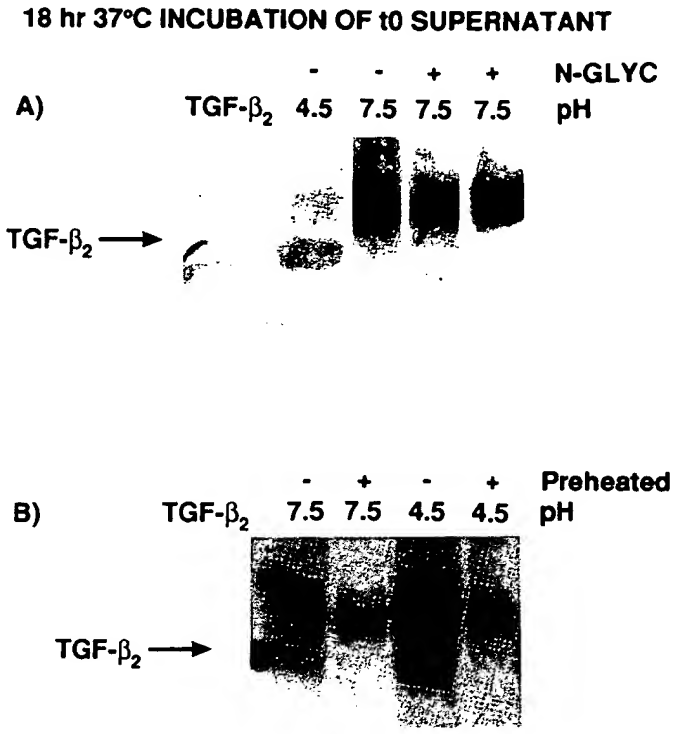

FIG. 7. Western blot study of TGFβ2-related decidual factor after treatment of t0 supernatant in vitro. **A)** From left to right: TGFβ2 standard, 25 ng (see arrow; note that downward slope is artifact at edge of gel); t0 supernatant incubated at pH 4.5 for 18 h; t0 supernatant incubated at pH 7.5 for 18 h; t0 supeernatant incubated at pH 7.5 with *N*-glycanase (Genzyme) or N glycanase (Sigma) for 18 h. **B)** From left to right: TGFβ2 standard, 50 ng; t0 supernatant incubated at pH 7.5 for 18 h; t0 supernatant incubated at 80°C for 10 min and then incubated at pH 7.5, 37°C, for 18 h; t0 supernatant incubated at pH 4.5; t0 supernatant incubated at pH 4.5 at 37°C for 18 h after an initial heating to 80°C for 10 min. Note that there is some variation in intensity of bands due to variation in resolubilization of concentrated lyophilized samples in the SDS sample buffer and due to the small amounts of biologically active molecules present. A higher concentration of standard was used, as detection efficiency of the rabbit anti-TGFβ2 was lower with later bleeds postimmunization.

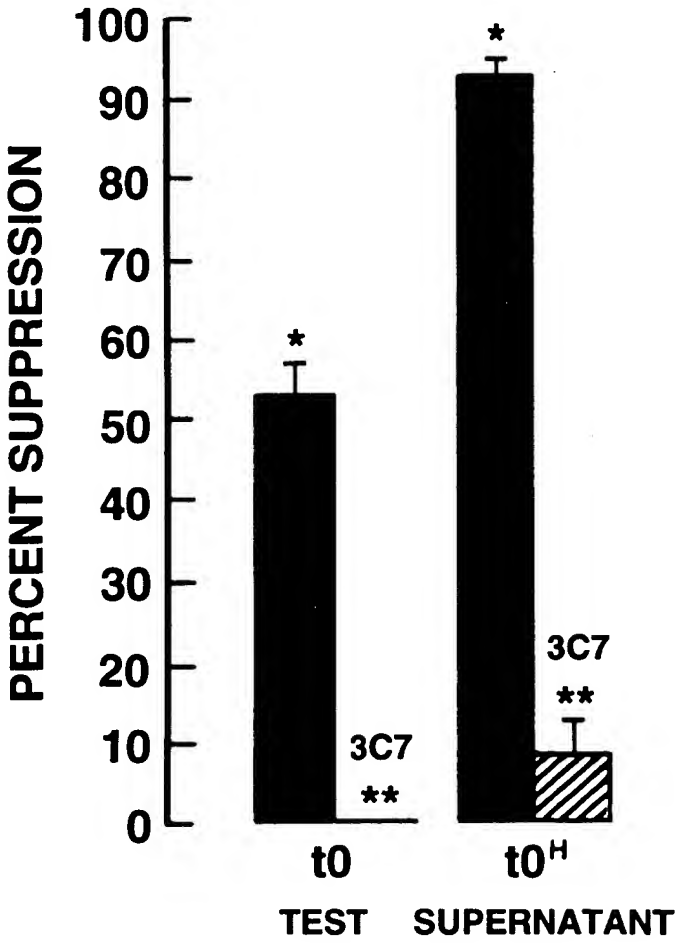

FIG. 8. Effect of monoclonal (3C7) anti-TGFβ2 antibody on immunosuppressive activity in serum-free supernatants; $t0^H$ = t0 supernatant heated to 80°C for 10 min. The 3C7 monoclonal is known to neutralize activity in FBS-containing supernatants whereas the 2G1 monoclonal binds but does not neutralize [1], and did not neutralize t0 or $t0^H$ activity (data not shown). All antibodies were used at a final concentration of 10 μg/ml and test supernatant at the dilution shown. *Denotes significant suppression; **significant abrogation of suppression.

TGFβ2 band was *not* generated in response to heat. Further, the shift in the band at pH 4.5 from high to low molecular mass was completely abrogated. Nevertheless, the heat-treated t0 supernatant retained its activity in the immunosuppression assay (Fig. 8), and the activity of both heated and unheated t0 supernatant could be neutralized by the 3C7 monoclonal anti-TGFβ antibody. The activity of heat-treated t0 supernatant was also retained when examined in the NRK colony-forming assay (data not shown).

Procollagen Synthesis Stimulation

A known effect of TGFβ1 is stimulation of collagen synthesis, which may then result in fibrosis [18]. The precise roles of the two mammalian TGFβ2 and TGFβ3 are not yet well understood. However, we have recently reported that TGFβ2 shares the ability of TGFβ1 to stimulate procollagen synthesis by fibroblasts in vitro [19]. Affinity column-purified decidual immunosuppressive activity was further purified by HPLC separation as described in *Materials and Methods*. The immunosuppressive activity of 1/10 and 1/20 dilutions was compared to the recombinant TGFβ2 standard curve, and by superimposition the relative amount of TGFβ present was determined. The purified immunosuppressive peak was also tested at 1/10 and 1/20 dilutions on fetal human fibroblasts [10]. Net procollagen synthesis was expressed as a percentage of maximum achieved with the TGFβ standard, and the result is illustrated in Figure 9A. The 1/10 but not the 1/20 dilution of the HPLC peak stimulated procollagen formation to a significant degree. However, when plotted as a function of TGFβ2 concentration in the assay, the potency of the decidual material was less than expected from the standard curve (activity of our recombinant TGFβ2 standard was not diminished by presence of the HPLC buffer [data not shown]). From the standard curve, the procollagen synthesis assay was more sen-

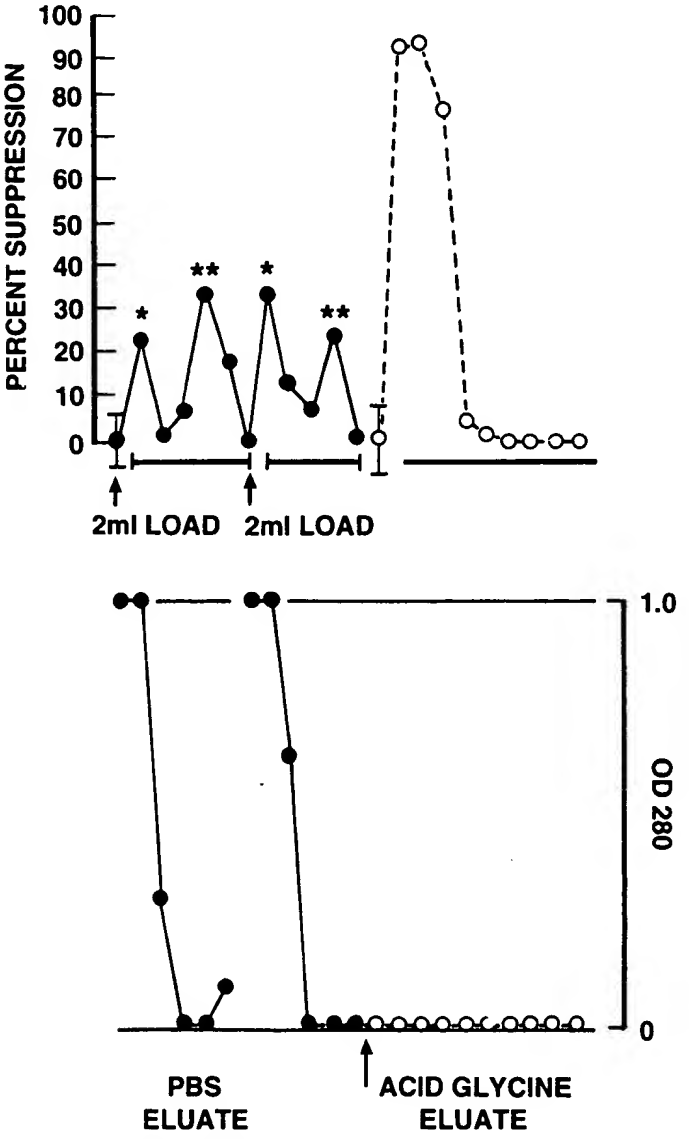

FIG. 5. Elution of decidual suppressor activity from 3C7 solid-phase affinity column. A t18 supernatant was used to saturate the column; * and ** indicate reproducible peaks of nonadherent activity. Solid circles: PBS eluate; open circles: acid glycine eluate.

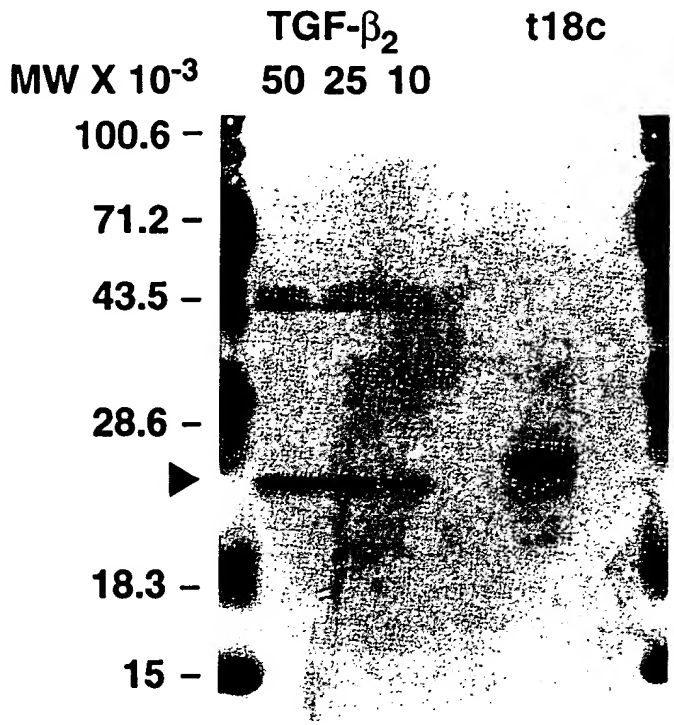

FIG. 6. Western blot analysis of TGFβ2-like material eluted by acid glycine from the 3C7 affinity column. The immunosuppressive activity peak eluted as shown in Figure 5 was collected and analyzed by Western blot assay using rabbit anti-TGFβ2 peptide. The arrowhead shows the location of the TGFβ2 standard (50, 25, or 10 ng). (Note that a lighter band at 43 kDa is not uncommon where higher concentrations of TGFβ2 standard are used.)

Further evidence that the molecules detected by Western blotting in Figure 3 were related to TGFβ2 was sought by affinity column separations using beads coated with the 3C7 monoclonal anti-TGFβ2 antibody. As can be seen in Figure 5, most of the activity in the t18 supernatant was bound, and the eluted activity showed both high and low molecular mass bands on Western blotting (Fig. 6). The activity (denoted by two asterisks) that was washed from the column by PBS pH 7.2 was also analyzed, and it appeared to be of the higher molecular mass form (data not shown); this could be explained by the column's having < 100% binding efficiency for the molecules. Surprisingly, little if any t0 supernatant activity could be recovered from the affinity column either in the wash phase or with acid glycine (data not shown), and this was reproducible with use of two separate preparations of 3C7-coated Reactibeads. It was not possible to separate the components of the doublet from each other or to separate the doublet from the lower molecular mass material by aqueous phase sieving HPLC in KCl-acetic acid buffer (data not shown).

Enzyme Treatment

The inactive TGFβ precursor is known to be glycosylated [14], and TGFβ2 possesses amino acids that could in theory act as glycosylation sites if accessible [18]. If lower molecular mass factors were produced by cleavage of carbohydrate (during incubation in vitro), such sites would need to be accessible to glycosylases. Several sets of experiments were done to try to modify the apparent molecular mass of the doublet shown in t0 supernatant in Figure 3. Figure 7A shows that *N*-glycanase treatment failed to shift the bands, but incubation in pH 4.5 buffer alone generated the lower molecular mass band; this made it impossible to determine whether endoglydosidase H had any effect (and indeed, no additional effect was seen; data not shown). When the t0 supernatant was first heat-inactivated at 80°C for 10 min and then incubated at pH 4.5, the result was as illustrated in Figure 7B. A conventional (i.e., authentic) 25-kDa

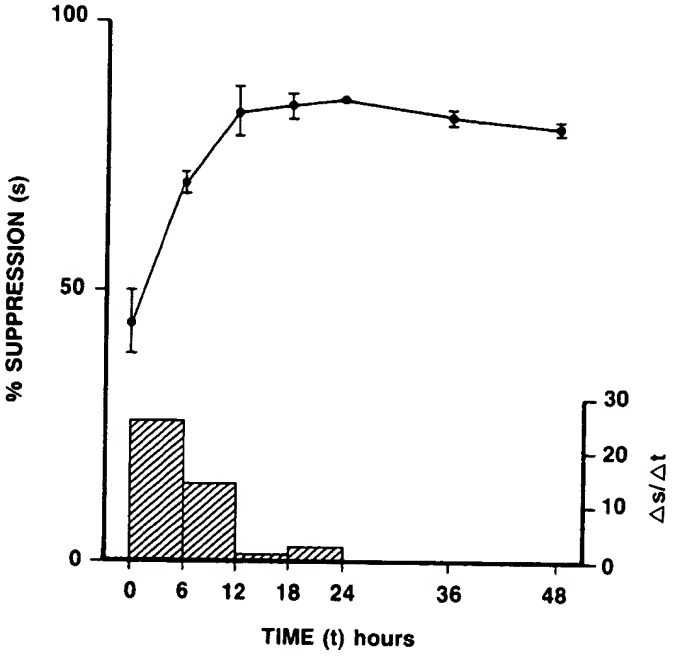

FIG. 2. Kinetics of release of suppressive activity from murine pregnancy decidua in serum-free medium. Increment in activity per unit time interval (6 h) shown as open triangle s/open triangle t. Error bars represent ± 1 SEM, 4 replicates per point.

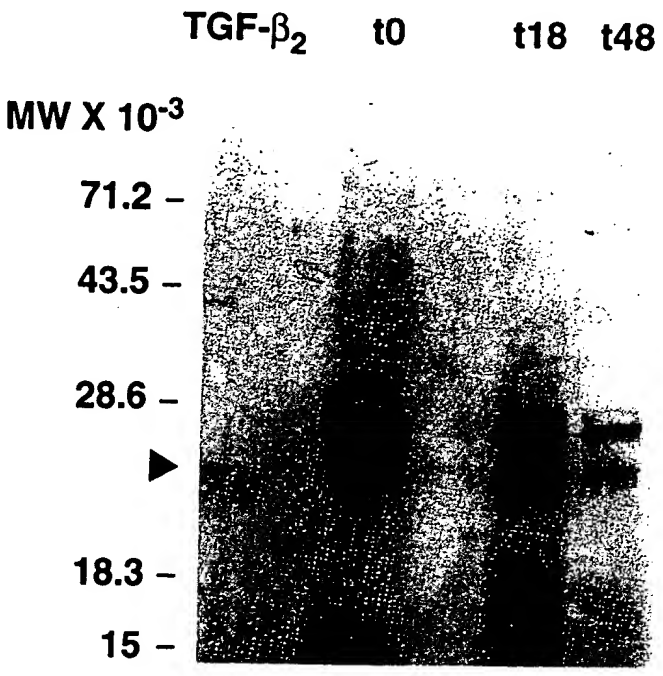

FIG. 3. Western blotting of serum-free medium for TGFβ2. t0 = Zero time wash fluid; t18 = supernatant after incubation for 18 h; t48 = supernatant after incubation for 48 h. Arrowhead denotes position of TGFβ2 standard (10 ng). MW × 10^{-3} is equivalent to molecular mass in kDa.

at 37°C. Activity was present at t0 and peaked at t18 (18 h). Thereafter there was some decrease in activity, but this was not statistically significant. There was no increase in activity when t0 supernatants were incubated at 37°C for 18 h in the absence of cells (data not shown). When the supernatants were analyzed by PAGE-Western blotting, a surprising result was obtained. Figure 3 shows that at t0, TGFβ2 antigenic activity was present as a doublet that migrated more slowly and at an apparent molecular mass greater than that of the 25-kDa porcine TGFβ2 standard. By 18 h of incubation, the lower molecular mass band previously described in the HPLC peak of 48-h decidual supernatants (prepared in medium with 10% FBS) [1] appeared, and the doublet became less intense. By 48 h, the doublet was even less evident, as was the lower molecular mass band; this is compatible with a small net decrease in activity as measured in the immunosuppression assay. These findings were confirmed in two additional independent experiments. With reference to the TGFβ2 and prestained standards, the doublet had an estimated molecular mass of 26 and 27 kDa, and the lower band had an estimated molecular mass of 20–23 kDa.

To determine whether bioactive TGFβ was being observed in the supernatants rather than a latent TGFβ that was being activated in vitro by low pH or enzymes (free or cell associated) [12–17] in the MLC-CTL assay, the supernatants were tested for the ability to stimulate NRK fibroblasts to form colonies in agar in the presence of EGF. It can be seen from the data in Figure 4 that active TGFβ was present at both t0 and t18. Activity was increased by exposure of the t0 supernatant to low pH, and this suggested that some latent TGFβ may also have been present.

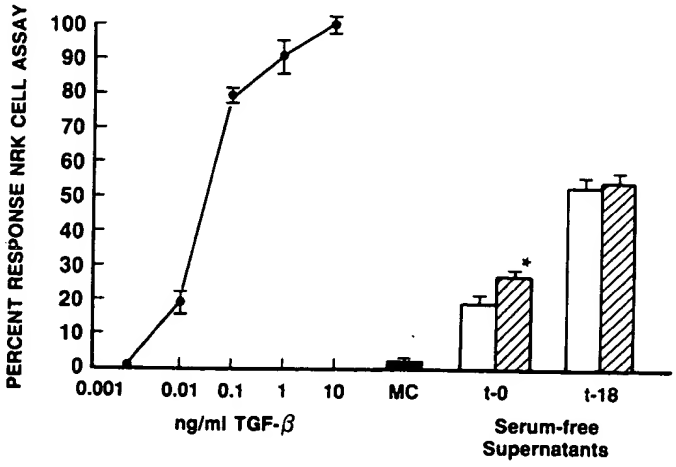

FIG. 4. Bioassay for TGFβ activity in serum-free media supernatants using NRK fibroblast assay (100% = 1.286 ± 0.023 o.d.; 0% = 0.116 ± 0.008 o.d. units). Time zero supernatants without treatment (open bar) or after acidification (hatched bar) to pH 2 and neutralization. *Denotes significant increase in suppressive activity, $p < 0.05$. Eighteen-hour supernatant (without treatment, open bar) or after acidification (hatched bar). Control medium (MC; solid bar) showed less than 0.01 μg/ml TGFβ (1.97 ± 0.8% OD) response; in an independent experiment, no TGFβ activity was generated by acid treatment of control medium. Purified TGFβ1 was used as the standard. Error bars represent ± 1 SEM, 4 replicates per point.